AD_____

Award Number: W81XWH-13-1-0078

TITLE: Can Exosomes Induced by Breast Involution Be Markers for the Poor Prognosis and Prevention of Postpartum Breast Cancer?

PRINCIPAL INVESTIGATOR: Virginia F. Borges, MD

CONTRACTING ORGANIZATION: The Regents of the Unviversity of Colorado
Aurora,CO 800415

REPORT DATE: July 2014

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2014	Annual	1 Jul 2013-30 Jun 2014

4. TITLE AND SUBTITLE

Can Exosomes Induced by Breast Involution be Markers for the Poor Prognosis and Prevention of Post-Partum Breast Cancer?"

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0078

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Virginia F. Borges, MD

E-Mail: Virginia.borges@ucdenver.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Regents of the Unviversity of Colorado
Aurora,CO 800415

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Breast cancers diagnosed up to six years after a completed pregnancy have been referred to as pregnancy-associated breast cancer or PABC. Several studies show that PABC frequently metastasizes, resulting in poor prognosis for the patient. Post-partum mammary gland involution is a necessary physiologic process required to return the lactation-competent gland to a non-lactating state. Accumulating evidence indicates that tissue-remodeling programs similar to wound healing are utilized to remodel the lactating gland to its post-partum state and that these programs are characterized by immune modulation. To move this work forward into the clinic, further understanding of the complexity between the tumor microenvironment and circulating factors that both influence the metastatic potential of these tumors and compromise the host immune response to the tumor are of great importance. It would also be ideal to have a circulating marker that would both identify women at risk for a postpartum breast cancer (PPBC), as well as, to assess the potential clinical benefit from novel therapies aimed to reduce the metastatic potential of PPBC. We have therefore undertaken this project to show , that exosomes with pro-metastatic cargo are released from the actively involuting gland, enter the circulation, and influence tumor-microenvironment interactions, immune escape, and the metastatic niche. In this proposal, our objectives are to determine, for the first time, whether exosomes with unique properties can be identified during involution, are likewise present in women with PPBC, and whether anti-inflammatory agent treatment mitigates their numbers, content, and or function. In year 1 of this project, we have successfully developed the new expertise to reliably isolate exosomes from animal plasma from tumor bearing and non-bearing hosts and have performed a rigorous methods comparison to ensure ideal assays are being subsequently performed. This methods development step was necessary due to the limited pre-existing data on exosomes from breast cancer animal models and serum plasma samples and their functional use.

15. SUBJECT TERMS
Young Women's Breast Cancer, Exosomes, postpartum breast cancer, immune suppression

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	32	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

Introduction:

Breast cancers diagnosed up to six years after a completed pregnancy have been referred to as pregnancy-associated breast cancer or PABC. Several studies show that PABC frequently metastasizes, resulting in poor prognosis for the patient.[1-7] We have proposed the postpartum involution-hypothesis to account for the high metastatic occurrence of PABC.[7,8] Post-partum mammary gland involution is a necessary physiologic process required to return the lactation-competent gland to a non-lactating state. Accumulating evidence indicates that tissue-remodeling programs similar to wound healing are utilized to remodel the lactating gland to its post-partum state and that these programs are characterized by immune modulation. [9-15] Thus, we have proposed that the involuting microenvironment, with its similarities to wound healing microenvironments, supports dissemination of tumor cells through immune suppression. In rodent models, we have identified the period of postpartum involution as a critical time point that promotes breast cancers progression, invasion and metastasis in animal models, data consistent with involution driving tumor progression. We have also shown that the promotional effects of involution are blocked by anti-inflammatory drugs given during the involution time frame.[16-17] To move this work forward into the clinic, further understanding of the complexity between the tumor microenvironment and circulating factors that both influence the metastatic potential of these tumors and compromise the host immune response to the tumor are of great importance. It would also be ideal to have a circulating marker that would both identify women at risk for a postpartum breast cancer (PPBC), as well as, to assess the potential clinical benefit from novel therapies aimed to reduce the metastatic potential of PPBC. We hypothesize that exosomes with pro-metastatic cargo are released from the actively involuting gland, enter the circulation, and influence tumor-microenvironment interactions, immune escape, and the metastatic niche. In this proposal, our objectives are to determine, for the first time, whether exosomes with unique properties can be identified during involution, are likewise present in women with PPBC, and whether anti-inflammatory agent treatment mitigates their numbers, content, and or function.

Body:

Statement of Work : Task 1 – Identify samples to be used in Aims 1-3 of the research and finalize regulatory approvals through DOD and HRPO. Anticipated timeline at grant initiation was **Months 1-3**

a. **Human Regulatory Issues** – the plasma from normal donors were collected under a specific protocol designed to have age matched controls for our ongoing research into the immune characterization and its impact on prognosis in YWBC and PPBC. This study has accrued over 80 normal donors. YWBC from newly diagnosed cases were obtained through an independent protocol (Immunology study) that collects blood, urine and breast tissues from our patient volunteers for the study of immune status, function and development of immune based treatment strategies against YWBC and PPBC. This study has accrued over 190 cases to date. Borges will select a subset of cases from both of these protocols to be balanced by age and for PPBC v non-PPBC status, biologic subtype, and stage among the cancer cases. We will aim for initially 20 normal and 20 YWBC cases to start, though these numbers can be flexed as required by the research results. There are 10 control and 10 drug treated cases of YWBC from our window of opportunity study (third prospective study) that have pre and post plasma samples ready to be used for this research. All three of these studies are approved by COMIRB, our local IRB, and two of the three (Immunology and WOO study) have already been reviewed in part by the DOD and HRPO for use of breast cancer tissue samples in our other DOD funded research. We will submit the research plan to the DOD and HRPO for approval on this research prior to initiation of the work. Of note, these samples are linked with fully annotated clinical data and tissue data from each subject to allow for clinical correlations.

RESEARCH ACCOMPISHED – YEAR 1 – The human specimens selected from the normal donor and cases of young women's breast cancer have been identified and the availability to adequate plasma confirmed for the initial testing of 20 normal and 20 YWBC cases, as well as the 10 control and 10 drug treated cases for aim 3. Work on these samples is pending completion of the regulatory process.

The regulatory process for approval of the use of these samples has been complicated. The complication stems from the fact that the samples were all collected under 3 separate research protocols that the PI of this grant is also the PI on. Each of those protocols clearly outlined and subjects gave consent for the use of those samples for the study of circulating factors (ie exosomes) that could contribute to immune suppression and different outcomes in young women's breast cancer and postpartum breast cancer. Initially, my local IRB felt that a new protocol was not required and that the use of these samples under a new grant required only a letter of acknowledgement to each of the protocols stating the added funding. This was performed in June 2013 (see timeline below). After being instructed by HRPO to create a new protocol, I had several meetings with my local IRB to determine what they would accept and whether the current protocols allowed for use of these samples as is or whether we would be required to go back and re-consent subjects. The manner in which the normal samples are collected does not permit re-consenting, as we do maintain any contact information with these subjects, and so after further clarification that these samples were being used for the exact intended purpose to which these subjects initially consented, we were granted waiver of re-consenting and could move forward with the submission of the re-use protocol.

<u>Detailed outline of research progress for regulatory approval is as follows:</u>

3/1/13 – email from USAMRMC, OPR, HRPO including forms for submission of use of samples as exempt.
4/1/2013 –PI phone conference call with Dr. Capell, head of COMIRB for discussion of use of samples while maintaining the link for future outcomes studies.
6/5/2013 – teleconference with USAMRMC, Brian Garland on plan for regulatory submission based on plan outlined by COMIRB.
Documents submitted with confirmation of receipt.
6/19/2013 Notification of project review through Melanie Frank
7/30/2013 Beginning of series of emails exchanges that the previously determined plan of submission was not preferred and consideration of a new protocol for use of these samples may be warranted.
8/28/2013 Conference call with Dr. Brosch, head of HRPO for confirmation of regulatory plan.
9/2013-April 2014 – several meetings with local COMIRB to clarify the change of regulatory plan and determine the process for a re-use protocol for the proposed sample use. April 2014, our local COMIRB made public it's "data and tissue use" algorithm that is identical to the publically available federal plan and we were given the green light to submit the re-use protocol with clear instructions on what needed to be included.
May 2014 – approval of the Exosome re-use protocol by the Scientific Review committee of the University of Colorado Cancer Center
July 22, 2014 – COMIRB APPROVAL OF THE EXOSOME RE-USE PROTOCOL
August 8, 2014 – Anticipated date of submittal of all DOD forms for HRPO final approval!

Dr. Borges and her team greatly appreciate all of the time and help provided by Brian Garland, Melanie Frank, Dr. Brosh and Dr. Warren Cappel of COMIRB to make it possible to move forward with this project. The locally approved re-use protocol is provided in the appendices.

b. Animal Approval Issues – The samples from our murine models of involution with and without tumor xenografts in the animals are all collected and available for immediate use. The samples were obtained under current IACUC approved protocols through co-Inv Schedin. We will also need to use up to 5 donor mice for the target BM derived progenitors to use in the immune functional studies as outlined in Aim 1. Full DOD review of the animal work will be requested prior to beginning the work.

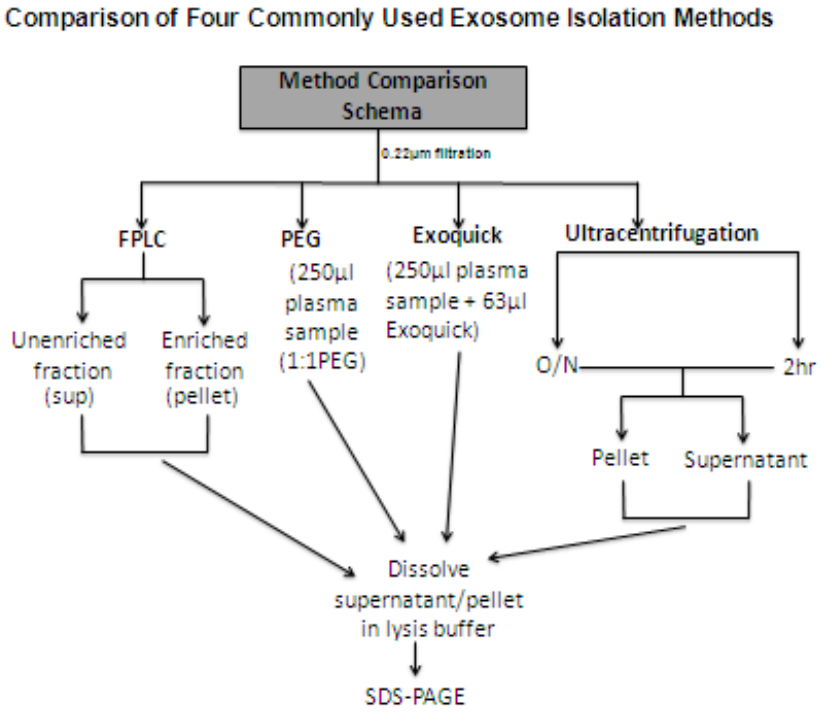
RESEARCH ACCOMPLISHMENT:
We initially had some confusion about the level of approval needed for this work and submitted a full AUCURO protocol. After further communication, we were granted the approval to proceed with all animal work on 11/26/2013, as only archival specimens are being used and no live animals being incorporated in to the research.

Task 2- Perform the exosome isolation from Aim 1 in the 4 animal cohorts: virgin and involution group and each with and without tumor xenografts and perform the characterization of the exosomes.
Months 3-12

a. **Exosome Isolation** –With training and support from the Graner and Eisenmesser labs, we will start with the mouse samples to ensure we have optimal isolation techniques. If inadequate exosomes are obtained with currents methods, we will change to PEG precipitation or use of currently available Exosome kits to ensure best methodology with our specific samples.
b. **Exosome characterization–** Identification of the exosomes through western blot, Nanosight analysis, size distributions and quantification analysis as described will be performed. Bouyant density determination and acetylcholinesterase activity determination and transmission electron microscopy will be performed.

RESEARCH ACCOMPLISHMENT:
As work on the animal samples began, we quickly realized that the available literature on exosomes and the optimal method of isolation and function use of these micro vesicles required additional investigation to determine what was going to be the optimal method or set of methods for use for the different aims of this project. For example, it is not clear that the same isolation techniques will have the same results in rodents versus humans and whether the same isolation technique will yield exosomes that are equally useful in subsequent functional assays, immune based assays and proteomics or if one isolation technique may be superior to others based on species or subsequent use.

Therefore, Task 2 has entailed a methodological comparison between the 4 most commonly used techniques: Ultracentrifugation (short 2 hour and long overnight), polyethylene glycol (PEG) based precipitation, forward phase liquid chromatography (FPLC) and Exoquick Reagent based precipitation. The schema for this methods comparison is represented below. The isolation techniques are assessed by a series of assays that identify the presence of microvesicles in the size range of exosomes, the overall protein content of the product derived by each method, electron microsopy for confirmation of the presence of



mircrovesicles and the identification of enrichment of the known exosome markers by Western Blotting. These markers incluse HSP 70 and the tetraspanins CD81, CD63 and CD9 as 4 of the most commonly used exosome markers.

Detailed methods are as follows:

Plasma Collection: Frozen blood plasma harvested from prior research protocols obtained from mice and rats were thawed as individual samples according to the standard procedures. The supernatant ad been stored at - 80°C in 250-300µl aliquots for future use and samples were within 3 -9 months of storage time.

Ultracentrifugation: Isolation of exosomes by UC was performed as follows. Briefly, blood plasma (250 µl) was diluted with 5-6 mL PBS and filtered through a 0.22µm syringe filter. The diluted plasma was then ultracentrifuged at 200000 × *g* for 2 hr. or overnight at 4°C. The resulting exosome pellet was resuspended in 100 µl PBS and an aliquot was stored at 4°C for Nanosight or electron microscopy. For Western Blot analysis, exosomes in the resuspended pellet or supernatant was lysed in RIPA buffer for 45 min at 4°C with rotation. The lysate was stored at - 80°C.

PEG based isolation: For exosome isolation by PEG based precipitation method, equal amounts of plasma and PEG 6000 were incubated overnight at 4°C on a rotating shaker followed by centrifugation at 10000 rpm for 20 minutes at room temperature. The resulting exosome pellet was resuspended in 100 µl PBS and stored at 4°C for Nanosight or electron microscopy. For Western Blot analysis, exosomes in the resuspended pellet or supernatant was lysed in RIPA buffer. The lysate was stored at - 80°C

Exoquick based isolation: Commercially available Exoquick Isolation Reagent (System Biosciences) was used for exosome isolation from plasma as well as melanoma cell line (A375) tumor conditioned media [supernatant] samples (as a positive control, supernatants provided from a research collaborator)

Plasma: For exosome isolation from plasma, thrombin plasma prep for exosome precipitation protocol was used according to manufacturer's instructions. Briefly, thrombin was used to pre-treat plasma to make it compatible with Exoquick exosome precipitation. The supernatant was used with Exoquick precipitation reagent (250µl sample + 63µl Exoquick) and incubated at room temperature for 30 min followed by centrifugation for 30 min at 1500g at 4°C. The resulting exosome pellet was resuspended in 100 µl PBS and stored at 4°C for Nanosight or electron microscopy. For Western Blot analysis, exosomes in the resuspended pellet was lysed in RIPA buffer. The lysate was stored at - 80°C.

Melanoma cell line tissue culture supernatant tumor conditioned media**:** For exosome isolation from tissue culture media (which was collected and filtered through 0.22µm filter), 250µl of media was incubated with 50µl Exoquick reagent overnight at 4°C followed by centrifugation for 30 min at 1500g at 4°C. The resulting exosome pellet was resuspended in 100 µl PBS and stored at 4°C for Nanosight or electron microscopy. For Western Blot analysis, exosomes in the supernatant or resuspended pellet was lysed in RIPA buffer. The lysate was stored at - 80°C.

Forward Phase Liquid Chromatography: For isolation of exosomes by size exclusion chromatography, 250-300µl of plasma sample was applied to a Superdex 200 prep grade column (Column volume 315ml). The column was eluted with PBS at a flow rate of 1ml/min and fractions were collected. The exosome fraction was concentrated using Amicon columns (Millipore) from a 2ml elution volume to 250 µl. The concentrate was used for Nanosight particle tracking analysis and lysed using RIPA buffer for Western blotting analysis.

Once the micro vesicles/exosome content is isolated, characterization/identification of the exosomes through western blot, Nanosight analysis, size distributions and quantification analysis and transmission electron microscopy were performed. We have opted to forgo the Bouyant density determination and acetylcholinesterase activity determination for now based on advice from our project exosome collaborators, Dr. Graner and Dr. Eisenmesser and corroborated by feedback received at a data presentation at a recent exosome symposium by external experts in the field of

microvesicles. Overall, these were thought to be redundant assays that would use up the exosomes and limit our ability to move forward with the functional assays planned.

Detailed methods of these assays are as follows:
Nanoparticle Tracking Analysis: The number of exosomes in the plasma samples, unenriched supernatant fractions and enriched pellet fractions was assessed using Nanosight LM10 nanoparticle tracking analysis (Nanosight Ltd., Amesbury, UK). Five video recordings were performed for each sample and batch process analysis function was used to measure the size of the vesicles. Batch process analysis integrates these five measurements and the average values were used to compare the size of vesicles/exosomes between samples.

Transmission Electron Microscopy: 3uL of sample was coated on the surface of Formvar-carbon grids and allowed to incubate for one minute at room temperature. The samples were then stained with 1% uranyl acetate and observed with a Philips transmission electron microscope operated at 80kV.

Western Blotting: Equal amounts of proteins from Exosome samples (20 µg) were loaded onto 10% Tris gels in. Gels were run at an initial voltage of 60V thought eh stacking gel and subsequently at 100 V through the resolving gel. Protein bands were transferred to PVDF membranes by wet transfer at 100V for 1 hr. The membranes were stained with Ponceau S solution to visualize protein transfer. The membranes were blocked with 5% non-fat dry milk in TBST and incubated with primary antibodies (Hsp70, CD81, CD63, CD9 System Biosciences Cat# EXOAB-KIT-1) at 4°C overnight. The membranes were washed in TBST and incubated in secondary antibody in non-fat dry milk at room temperature for 1hr. The protein bands were visualized using Pierce ECL Kit.

Results:

We initially cultured the murine mammary cell lines D20R and D2A1 (cell lines with reportedly a "less aggressive and more aggressive" pattern of behavior derived from a balb/c genetic background) and obtained tumor conditioned media in both the context of standard use cell line media and cell line media without serum, as we were concerned that the serum content of the media could lead to the false identification of exosomes from the serum rather than the cell line. A cell line was chosen, as it is an easier replicated resource and would be a more certain source of exosomes than plasma samples from animals for our initial assay optimization. Figure 1 demonstrates that indeed the presence of serum in the media did lead to an increase in the identification of exosomes, as seem in the increase of selected marked HSP 70, CD81, CD63 and

Figure 1: Exosome Protein Markers are Expressed by Mouse Mammary Tumor Cell Lines

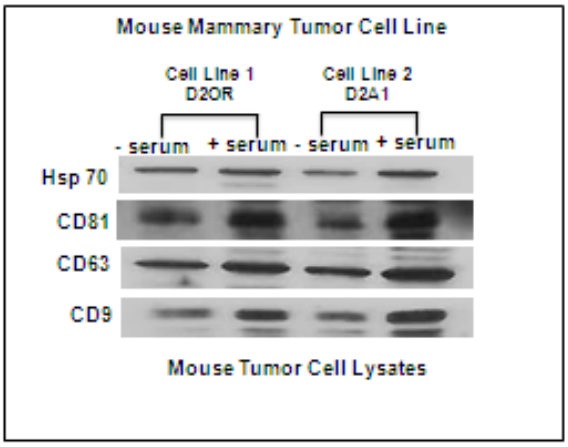


Figure 1: Exosomes protein markers are expressed by mouse mammary tumor cell lines. Cell lysates were collected and analyzed for exosome protein expression by Western Blotting for exosome markers Hsp 70, CD81, CD63 and CD9. Both cell lines D2OR and D2A1 express the commonly known exosome markers. Notably, the expression of all these four markers seem enhanced in the presence of serum compared to cells cultured in the absence of serum.

CD9 for both tumor supernatants. Based on these results, we have performed all subsequent assays in the absence of serum or in very low serum concentrations to minimize the contamination of exosomes.

Figure 2: Microvesicle Enrichment in Tissue Culture Supernatants

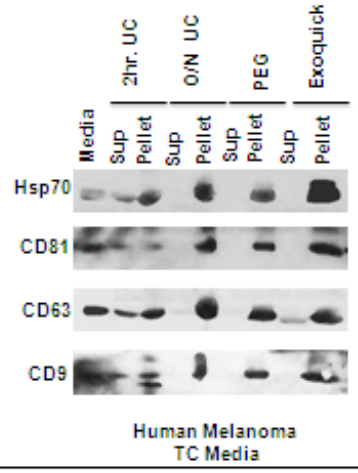


Figure 2: Microvesicles are enriched in the pellet fractions by ultracentrifugation, PEG and Exoquick based isolation methods. Tissue culture media supernatants were collected, filtered through a 0.22µm filter and subjected to 2hr. or overnight (O/N) ultracentrifugation (UC), polyethylene glycol (PEG) based precipitation or exoquick reagent based isolation. The supernatant(sup) and pellet were analyzed for exosome protein expression by Western Blotting for exosome markers Hsp 70, CD81, CD63 and CD9.

We then began our comparison of the 4 methods for exosome isolation. Again, we started with supernatants from a melanoma cell line as a reproducible resource that is also essentially a positive control to make sure our methods of detection were accurate. The rationale being that if we could not reliably find exosomes from these supernatants, we would have significant further assay optimization work to perform before we would be realistically able to expect to identify them from the plasma of tumor bearing and non-tumor bearing hosts. Three of the 4 tested methods were able to reliably demonstrate the enhanced concentration of exosomes in the enrichment product or "pellet". The FPLC technique proved very problematic from the outset in terms of both logistical issues with access to the equipment but also in terms of the volume of plasma needs for the isolation technique and the extremely low quantity of microvesicles able to be identified. The blots on the westerns run on these samples are so minimal that only significant over exposure allows them to be seen at all, and therefore that data in not presented in comparison with other assays. Figure 2 is a representative western blot of the isolation techniques of ultracentrifugation with two time frames of 2 hour and overnight (O/N), PEG isolation and Exoquick kit method. It shows the progressive enrichment of micro vesicles expressing HSP 70 and the tetraspanins, CD81, CD69 and CD9. Of note, these markers are determined from extensive review of the literature. The tetraspanin markers do not have a known function as part of their presence on exosomes at this time. Figure 2 shows the level of marker detection from the base "media" or supernatant pre-isolation in the column on the far left and then the enrichment across each of the markers for the out-product or "pellet" of the enrichment in comparison to the left-over supernatant after the enrichment process or "sup". All 3 methods were able to demonstrate isolation, though there was a noticeable pattern across runs that the overnight or O/N ultracentrifugation had superior product than the 2 hour UC process. An additional step was to repeat these experiments on fresh supernatant versus supernatant that had been stored for 3-9 months, which more accurately will reflect our plasma samples to be used in this project., with no differences identified between the fresh and frozen samples. After these results, we felt confident to move into our rodent samples and test the plasma samples from tumor-bearing animals across the methods. From these samples we also incorporated the steps of identifying the mean size of the micro vesicles isolated to ensure we were in range of expected exosomes size and to see if any variability if the content of size was noted across the techniques. We also analyzed the protein concentration to see the overall estimated amount of exosomes being pulled down by the methods. This is a crude analysis as other proteins pulled down by the methods will also be present, but it allows a baseline level of method comparison at an adequate level of quantitation. The results demonstrated that all 4 tested methods enriched for micro vesicles in the expected range from 50 -200nm for exosomes. Across the ultracentrifugation methods, PEG and Exoquick methods, there was no significant difference on the mean or mode size of the isolated vesicles. The FPLC data was unclear, as the results were not consistent

with what the starting media results provided and are considered not reliable for accurate size quantitation and raised the issue of possible contamination of the specimen while being run on the FPLC column. The protein concentration estimation showed that all methods other than FPLC had an adequate level of protein present in the end product or pellet, with O/N ultracentrifugation again outperforming the 2 hour technique. FPLC had a minimal detectable protein concentration, suggesting in adequate enrichment or loss of sample. Western blot for exosome markers confirmed that we can readily identify HSP 70, CD81 and CD63 as successfully enriched exosome product from all methods other than FPLC.

It is reported in the literature that there are isoforms of CD63 and across replicates, we have seen single versus double or multiple bands present, the significance and pattern of which we are still investigating. We also noted that the CD9 tetraspanin was not regularly identified in the plasma samples from the animals. Not all published papers include this marker, so we are investigating of this could be a product of the tumor or animal or species or need for more antibody optimization across the samples. Figure 3 shows the data from these experiments, with 3c a representative Western from the replicates. In summary, we believe we identified that the PEG and overnight ultracentifugation were the most reliable for delivery of micro vesicles in the desired size range, with good protein content and will most reliable enrichment for the exosome markers in stored frozen plasma from tumor bearing rodents. The FPLC was an ongoing problem across all attempts at using this method, as the data demonstrated. We were also subsequently informed by our collaborator, Dr. Eissenmesser, that the column used for the FPLC isolation

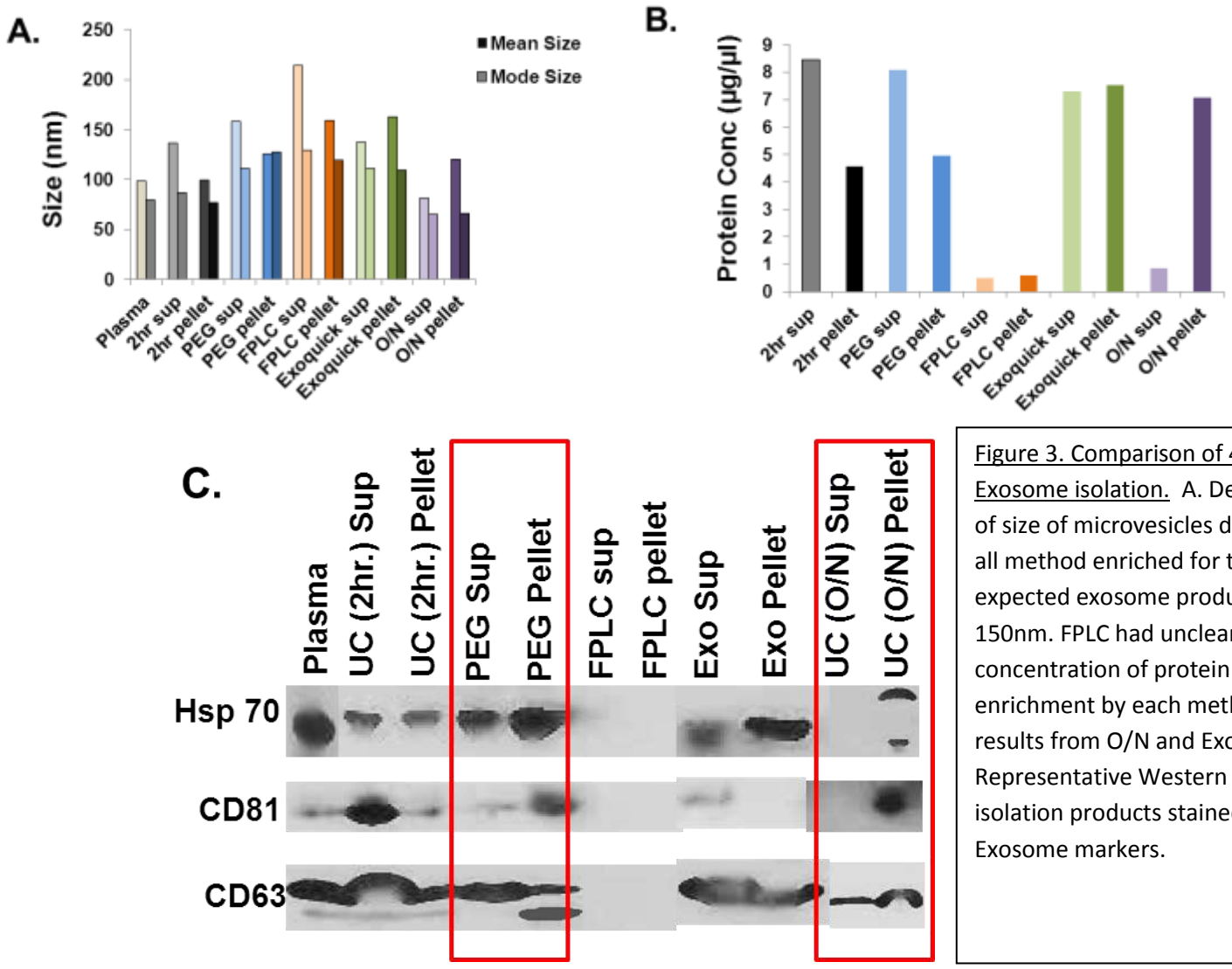
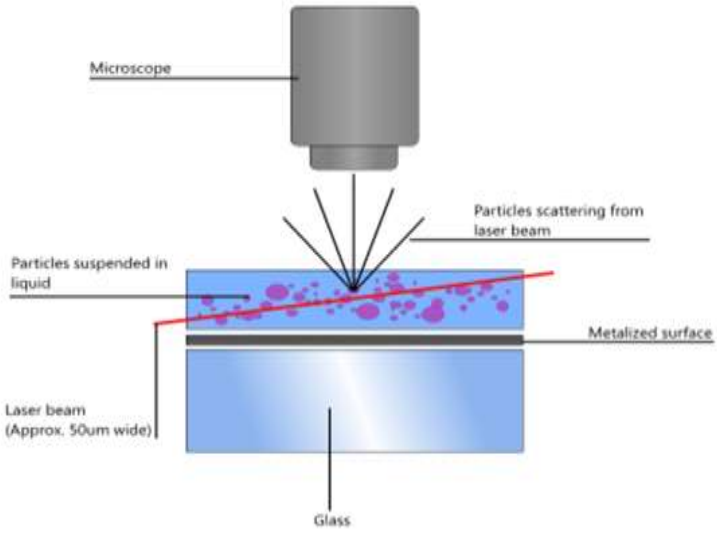
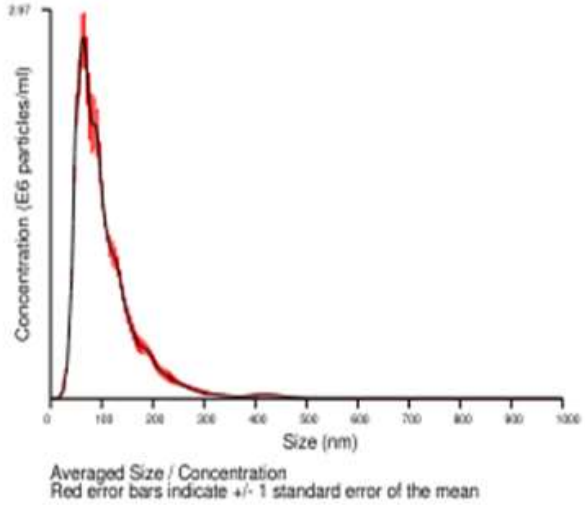


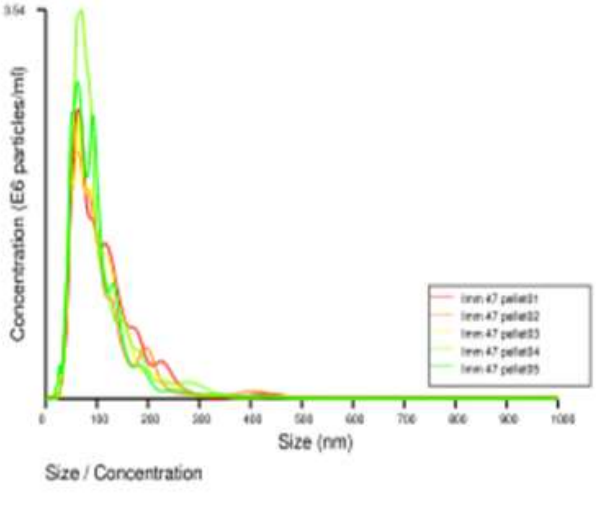
Figure 3. Comparison of 4 methods of Exosome isolation. A. Determination of size of microvesicles demonstrates all method enriched for the size of expected exosome product of 50-150nm. FPLC had unclear results. B. concentration of protein after enrichment by each method with best results from O/N and Exoquick. C. Representative Western on the isolation products stained for known Exosome markers.

Analysis of samples by nanosight and electron microscopy has been performed. Because we are in the phase of methods optimization and these are not the full formal experimental cohort samples, only selected samples have been used for follow through into the characterization steps. Representative nanosight data is shown below in Figure 4. Nanosight is the increasing utilized technique for confirm the presence of micro vesicles/exosomes. The working principle of the Nanosight is very similar to how a flow cytometer works. The fluidics associated with the instrument send the particles as a stream where they are hit by a laser and you get scatter data which can tell you the size of these particles. The difference from flow cytometry is that the nanoparticle tracking system tracks these particle and records videos of their motion based on the scatter data that it collects. You can see the motion of these particles in the lower left picture of Figure 4 where the particle was tracked along the length of the red line. The smaller tracks you see here could be either the starting or the end of the track length or they may not be real particles. Based on the

Figure 4: NANOSIGHT (Nanoparticle Tracking Analysis)







parameters set, if the particle cannot be tracked for a certain number of frames, it is not considered a real particle. Once all these videos are taken, the data is combined to give a graph like the one shown in the bottom R of Figure 4. As expected, the peak is at the size for 100nm particles with the range of the area under the curve being from 50-200nm, consistent with exosomes. The graph of peaks to the right of the two graphs in Figure 4 show a script that

was run where each sample was run as 5 times, each video being a recording of 5 videos, so an overall of 25 videos are combined and the green line graph is generated and the red bars show the std. deviation. Thus, the replicates presented demonstrate the reproducibility of the analysis. The peak Electron microscopy picture in not included but did show reliable identification of spheres present consistent with exosomes. At present, for the murine work, we have identified overnight ultracentrifugation to be our preferred method. Though it is not demonstrably better than the PEG or Exoquick methods, it is considerably more cost effect than the Exoquick and does not introduce any additional reagents into the isolation that could prove problematic when we perform the subsequent functional analyses and proteomics. Lastly, to date our optimization of methods has included the analysis between plasma samples obtained from virgin animals with and without tumor present to identify any differences in the presence of absence of tumor with the main chosen method of O/N UC.

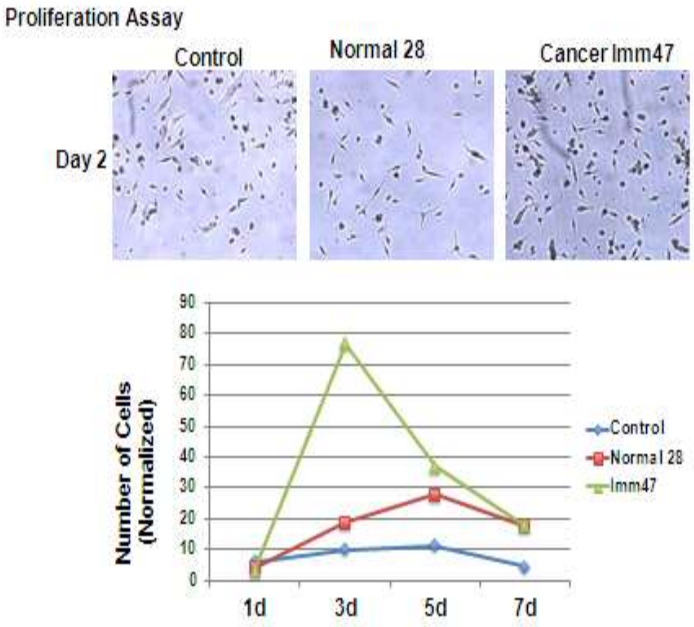
Task 3 – Perform the characterization of the proteome content of the 4 animal cohorts of exosomes
Months 12-24

a. In depth quantitative proteomic analysis on the exosomes identified from the virgin, involution, virgin with tumor and involution with tumor plasma samples will be performed according to our published protocols and methods as outlined in the grant. This work will be performed in the Hansen lab, with collaborative input from Eisenmesser and Graner as needed.

RESEARCH ACCOMPLISHMENT:
At our exosome project meeting in May, the data on our isolation techniques were reviewed by all grant members and collaborators with excellent feedback provided. Dr. Hansen has signed off on the quality of our microvesicle/protein isolation technique with the overnight centrifugation as being of high enough specificity to permit moving forward with the proteomics analysis. Therefore, the first set of rodent samples from tumor plasma samples are in the Hansen lab undergoing proteomic analysis. Results are pending at the time of this submission. Based on these first results, we will move forward with adding samples from each of the parity subgroups and the non-tumor virgin and involution samples as well.

Task 4 –Perform functional analysis of the exosomes from the animal cohorts on tumor cell behavior using the target cell line of MCF10.DCIS human cell line for proliferation, migration and alteration of tumor morphology and apoptosis rates in 3D culture models.
Months 6-18

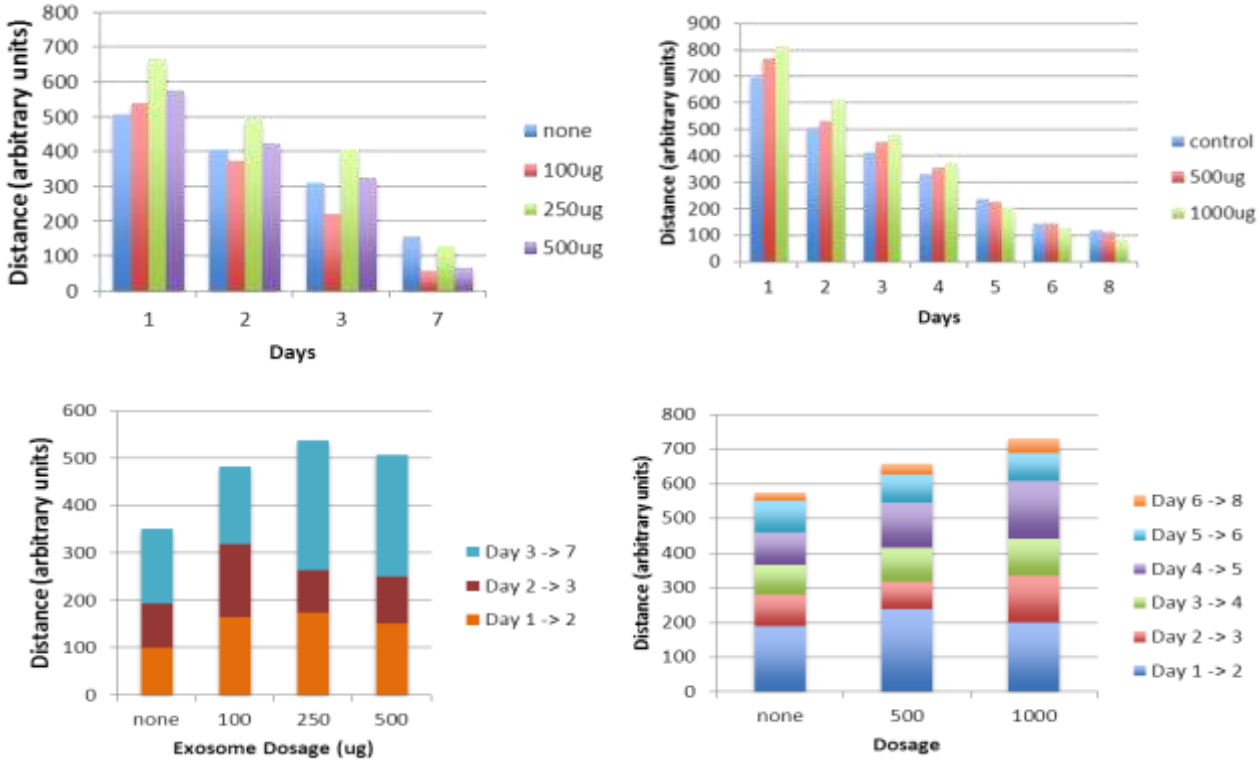
Figure 5



RESEARCH ACCOMPLISHMENT:
After beginning the work on this aim, we learned that exosomes can express Class I antigens. Therefore we modified the assay to use congenic animal mammary cancer cell lines, rather than the DCIS.com cells as the read out of the effect of exosomes on tumor behavior to avoid this possible confounding issue. We are

in the process of optimizing the proliferation and migration assays and preliminary data is presented. Proliferation assays were performed with D241 congenic murine cell line after demonstrating reliable degrees of proliferation and migration in the absence of any additives at various concentrations of plating. Cells were plated on a 12-well plate, incubated at 37degrees and at identified time points were counted using a 1:2 dilution (20uL) from a 1mL volume using a hemocytometer. Initial assays were performed with tumor-bearing animals to maximize results. Initial results comparing 0 to 250ug of exosome content [protein content as surrogate measure for exsomes] did not detect a significant difference over a 7 day assay. We then moved on to adding exosomes at differing "doses" of 0, 250ug, 500ug and 1000ug of protein added and identified that the "dose" of 500ug appeared to have the most robust and reliable increase in proliferation with a notable decline at the higher level. We then moved on to compare normal, non-tumor bearing plasma to tumor plasma and initial results are shown in Figure 5. the proliferative response of the plated tumor had a more robust level of proliferation by day 3 when co-cultured with exosomes derived from a sample from a tumor-bearing host [Cancer Imm 47] versus a non-tumor bearing host [Normal 28] versus control plated cells without exosomes present. The tumor cells rapidly outgrow their media after day 3 and cell death is visibly apparent, which is not unexpected in the no serum conditions being used. Further optimization of low serum conditions and difference cell numbers are planned for ongoing optimization of the assay. Additional replicates are pending to permit for statistical analysis. More robust measure of proliferation, such as crystal violet assay, will be incorporated, as well as a survival assay with caspase glow analysis. Preliminary data on tumor migration also shows an apparent exosome "dose dependent" response that is still under development with further titrations planned before assay will be considered ready to go for experimental samples. As shown in Figure 6, initial

Figure 6. Migration of mammary tumor cells with exosome exposure



experiments showed a higher level of proliferation at an exosome dose of 250ug. However, in this assay, the plates were photographed and notable cell death and plate overgrowth appeared to be occurring with the 500ug dose level. Therefore, assays were set up with doses of 500 and 1000 ug. There appears to be an increased effect with higher doses of exosomes initially, that is then lost as the tumor cell line reaches confluence. We have also charted the data by distance closed by day of migration to demonstrate that in the setting of exosome exposure, the tumor cells migrate for longer. Further work is needed to know that we have both the best cell line and concentration of baseline plating and the best media/serum condition to provide for optimal readout without confounding the exosomes effects

and the best method of "dosing" the tumor cells with exosomes. We also plan to incorporate Matrigel coating on the plates, as is very commonly used but not always incorporated in the published exosome literature. Once these issues are resolved, we will be able to rapidly move forward with the experimental cohorts and complete this task. Tumor morphology and apoptosis in 3D culture models is pending.

Task 5- Perform functional analysis of the exosomes from the animal cohorts on their ability to induce immunosuppressive cells and drive the immune function of monocytes, Tregs, MDSC and effector T cells to a pro-tumor, immunosuppressive state.
Months 12-24

RESEARCH ACCOMPLISHMENT:
We have begun Task 5 and are in the process of optimizing the immune function analyses for the function of monocytes, Tregs, MDSC and effect T cells in the setting of exposure to exosomes from non-cancer bearing versus cancer bearing hosts. Insufficient data are ready for presentation for this report, but we are on time for the start of this task and have no difficulties encountered yet.

All work from here forward in the statement of work has not yet begun as the approval from HRPO for the use of our human samples is pending. Overall, we are still on target with the timeline of research work for this 3 year project and anticipate no difficulties in continuing to complete the aims as outlined and in the timeline outlined.

Task 6- Exosome isolation and characterization from the human samples from normal young women and YWBC samples
Months 6-18

a. Using our optimized isolation and characterization techniques from Task 1, we will go forward with the first set of human samples to include 20 normals and 20 cases of YWBC as outlined above. Samples can be expanded or adjusted for their clinical characteristics as determined by the results.

Task 7 –Proteomic analysis of the exosomes from normal and YWBC cases.
Months 12-30

a. Work to be performed in the Hansen lab, similar to as outlined for the animal samples in Task 3
b. Proteome content will be compared between the animal groups and the YWBC samples by parity status to correlate for similar content that may be unique to the effects of postpartum involution on PPBC. Unique proteins identified as matched between involution, involution with tumor and human PPBC will be explored in greater detail for functional characteristics and insight into possible mechanism of functioning.

Task 8 – Perform the functional analysis of the exosomes from unaffected young women and YWBC on tumor cell behavior using the target cell line of MCF10.DCIS human cell line for proliferation, migration and alteration of tumor morphology and apoptosis rates in 3D culture models. **Months 18-24**

Task 9- Perform functional analysis of the exosomes from the animal cohorts on their ability to induce immunosuppressive cells and drive the immune function of monocytes, Tregs, MDSC and effector T cells to a pro-tumor, immunosuppressive state.
Months 18-24

Task 10 – Isolate exosomes from the samples collected pre and post drug intervention with either celecoxib or nil on our completed WOO human clinical trial.
Months 18-33

a. Given the unique and precious nature of these samples and the effort it would take to recreate them, we will wait until we have completed significant work on the animal and human samples from the other studies and feel secure on our techniques and methods with isolation and functional read outs.

Task 11-Perform the proteomic analysis on exosomes from the WOO clinical trial samples.
Months 24-36

a. We will continue with Task 11, using techniques optimized through Tasks 3 and 7.
b. We will focus on those changes to the proteome content that correlates most with unique characteristics found in the involution, involution with tumor and PPBC samples.

Task 12- Analyze the human exosome data (quantity, proteome content and functional data) from the YWBC samples and the WOO clinical trial and compare with known clinicopathologic parameters and study outcomes for clinical relevance
Months 24-33

a. The results from the exosomes from the WOO trial samples pre and post on each of the 20 cases will be correlated with the pre and post Ki-67 index and its change across the study drug intervention time period and by cases status description of PPBC versus non-PPBC.
b. Correlations with other known histologic parameters of breast cancer (stage, biologic subtype, age [young v very young] will be performed as well for all the YWBC cases
c. Identification of any specific effects (Reversal) of the anti-inflammatory drug intervention assigned on the exosome quantity, proteome content or functioning with respect to the overall population and by the cohorts of interest (PPBC and non-PPBC). Correlations with biologic subtype of breast cancer will be explored in a hypothesis generating manner**.**

Task 13 – Presentation of data accumulated to date at DOD Era of Hope Conference
Month 24(approximately)

Task 14 - Preparation of manuscripts from the finalized data from the completion of Aims 1-3
Months 24-36

Task 15 –Preparation and submission of ongoing grants based on outcomes from this work to continue to develop treatment and prevention strategies against the postpartum effect of involution on young women's breast cancer
Months 24-36

Key Accomplishments:

For year one of this award the key accomplishments to date include:

1. Detailed methodologic comparison of available exosome isolation techniques with optimization of techniques to our samples.

2. Development of lab expertise in the relatively nascent field of micro vesicles and exosomes with presentation of data at an exosome symposium where our data was highly received for both its comprehensive approach and unique findings.

3. A year of lab training for a new post-doc, Dharanija Rao, who was recruited just after graduation to work on this project and has accomplished her first poster presentation at a national meeting and a very well received presentation in the Cancer Biology program at our institution. Presentation at the forthcoming national microvesicle meeting in Boston, October 2014 is planned.

4. Initial optimization steps for proliferation, migration and immunology assays for the work outlined. Importantly, it must be emphasized that the field of exosomes is very early and there are not steadfast, reliable protocols available for our use, each step is a novel assay and novel optimization strategy that we are developing specifically for our sample sources.

5. Research education for a summer college student who has spent 6 weeks in the lab for a research experience as part of the University of Colorado Cancer Center Summer Student Research Program.

Reportable outcomes:

1. A methods paper outlining our work as presented above is in preparation. We intend to compliment the present data with the human data to be obtained as soon as HRPO approval is gained to provide a unique body of work to the microvesicle field.

2. The following presentations have occurred to date:

    a. Exosome Expo, Denver, Colorado -May 2014, Poster presentation, Title: Characterizing Exosomes in Circulation in Rodent Models

    b. CANCER BIOLOGY PROGRAM, University of Colorado Cancer Center, Seminar Series, Oral presentation, May 2014, Title: Characterizing Exosomes in Circulation in Rodent Models

Conclusion:

In summary, we have begun the identification of exosomes from rodent plasma samples obtained from virgin non-tumor bearing and tumor-bearing animals. We have identified reliable methods for isolation of exosomes that will suit our ongoing work and have begun the development and optimization of function assays to detect the change in tumor behavior after exposure to exosomes from these animal cohorts. Changes incorporated into the work so far include modification of the cell line read-out for the functional assays to avoid any xenograft affects that could arise from the presence of class I antigens on the exosomes. We have moved forward with the preliminary proteomic studies based on satisfactory exosome isolation techniques. We look forward to the next two years of this project as we can now move forward to the experimental comparisons between nulliparous and postpartum hosts with and without tumors present to identify the difference in exosome present and/or their differing functional charateristics. We foresee that this work will lead to a novel potential target for breast cancer, if these exosomes do indeed alter tumor aggression and host immune suppression. Moreover, their unique identification may serve as a "liquid PET scan" to help identify women at risk for metastatic disease through incomplete clearance or re-appearance of tumor-associated exosomes over time after treatment.

References:

1. Lambe M, Hsieh C, Trichopoulos D, Ekbom A, Pavia M, Adami HO: Transient increase in the risk of breast cancer after giving birth, The New England journal of medicine 1994, 331:5-9
2. Robertson C, Primic-Zakelj M, Boyle P, Hsieh CC: Effect of parity and age at delivery on breast cancer risk in Slovenian women aged 25-54 years, International journal of cancer 1997, 73:1-9
3. Albrektsen G, Heuch I, Hansen S, Kvale G: Breast cancer risk by age at birth, time since birth and time intervals between births: exploring interaction effects, British journal of cancer 2005, 92:167-175
4. Albrektsen G, Heuch I, Kvale G: Further evidence of a dual effect of a completed pregnancy on breast cancer risk, Cancer Causes Control 1996, 7:487-488
5. Albrektsen G, Heuch I, Tretli S, Kvale G: Breast cancer incidence before age 55 in relation to parity and age at first and last births: a prospective study of one million Norwegian women, Epidemiology 1994, 5:604-611
6. Colditz GA, Frazier AL: Models of breast cancer show that risk is set by events of early life: prevention efforts must shift focus, Cancer Epidemiol Biomarkers Prev 1995, 4:567-571
7. Lyons TR, Schedin PJ, Borges VF: Pregnancy and breast cancer: when they collide, Journal of mammary gland biology and neoplasia 2009, 14:87-98
8. Schedin P: Pregnancy-associated breast cancer and metastasis, Nature reviews 2006, 6:281-291
9. Schedin P, Strange R, Mitrenga T, Wolfe P, Kaeck M: Fibronectin fragments induce MMP activity in mouse mammary epithelial cells: evidence for a role in mammary tissue remodeling, J Cell Sci 2000, 113 (Pt 5):795-806
10. Schedin P, Mitrenga T, McDaniel S, Kaeck M: Mammary ECM composition and function are altered by reproductive state, Molecular carcinogenesis 2004, 41:207-220
11. Schedin P, O'Brien J, Rudolph M, Stein T, Borges V: Microenvironment of the involuting mammary gland mediates mammary cancer progression, Journal of mammary gland biology and neoplasia 2007, 12:71-82
12. O'Brien J, Schedin P: Macrophages in breast cancer: do involution macrophages account for the poor prognosis of pregnancy-associated breast cancer?, Journal of mammary gland biology and neoplasia 2009, 14:145-157
13. Bemis LT, Schedin P: Reproductive state of rat mammary gland stroma modulates human breast cancer cell migration and invasion, Cancer research 2000, 60:3414-3418
14. Clarkson RW, Wayland MT, Lee J, Freeman T, Watson CJ: Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression, Breast Cancer Res 2004, 6:R92-109
15. Stein T, Morris JS, Davies CR, Weber-Hall SJ, Duffy MA, Heath VJ, Bell AK, Ferrier RK, Sandilands GP, Gusterson BA: Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3, Breast Cancer Res 2004, 6:R75-91
16. O'Brien J, Lyons T, Monks J, Lucia MS, Wilson RS, Hines L, Man YG, Borges V, Schedin P: Alternatively activated macrophages and collagen remodeling characterize the postpartum involuting mammary gland across species, The American journal of pathology 2010, 176:1241-1255
17. Lyons TR, O'Brien J, Borges VF, Conklin MW, Keely PJ, Eliceiri KW, Marusyk A, Tan AC, Schedin P: Postpartum mammary gland involution drives progression of ductal carcinoma in situ through collagen and COX-2, Nat Med 2011, 17:1109-1115

Appendix: Clinical protocol for sample use - current COMIRB approved version 1

Protocol Title: "Can exosomes induced by breast involution be markers for the poor prognosis and prevention of post-partum breast cancer?": Bio-sample re-purposing protocol for the ongoing study of immunosuppression in young onset breast cancers.

Coordinating Institution: University of Colorado Anschutz Medical Campus

Study nickname: Exosome re-use protocol

Principal Investigator:
Virginia F. Borges, MD, MMSc
Associate Professor
Director, Young Women's Breast Cancer Translational Program
Division of Medical Oncology
University of Colorado Anschutz Medical Campus
12801 E 17th Avenue, Room 8112
Aurora, CO 80045
virginia.borges@ucdenver.edu

Study Coordinator:
Emily Rozzo, BS
Translational Research Associate
Young Women's Breast Cancer Translational Program
Emily.rozzo@ucdenver.edu

Study Synopsis:

Title: "Can exosomes induced by breast involution be markers for the poor prognosis and prevention of post-partum breast cancer?"

Clinical Development Phase: Translational study for the re-purposing of samples obtained under prior research protocols [COMIRB 08-1040, 09-0583 and 11-0357] for use under new funding source DOD/CDMRP Proposal Log Number BC121782, Award Number W81XWH-13-1-0078

Study Overview: We will utilize previously collected blood samples from three prospective, translational bioanalysis studies aimed at investigating immune suppressive parameters in the systemic circulation and tumor/stromal microenvironment at diagnosis and/or time of subsequent recurrence in women with breast cancer and normal control subjects. For this new protocol, we will specifically investigate a recently identified potential cause of immune suppression in cancer called exosomes. These are circulating microvesicles released into the extracellular environment that have been identified in the plasma of cancer patients and correlated with poorer prognosis and survival. At the time of initiation of all three clinical trials, enrolled subjects gave consent for use of their blood for the study of circulating factors that may be causative or related to tumor-induced immune suppression in young women's breast cancer. The knowledge and technology now exists for us to move forward with this planned aspect of the research through this in depth proposal to study circulating exosomes from women with young onset breast cancer, with young onset breast cancer who were exposed to immune modulating anti-inflammatory drugs and in women who have not been diagnosed with breast cancer as controls.

Objectives/Specific Aims:

Aim 1. Characterize the circulating exosomes present in YWBC. Determine the type of circulating exosomes, protein content and function between unaffected young women and age-matched newly diagnosed cases of YWBC and correlate these findings with parity status [post-partum breast cancer (PPBC) or non-post-partum breast cancer (non-PPBC) and known prognostic clinical tumor characteristics.

> **Innovation, Rationale and Impact:** The identification of increased or unique circulating exosomes in primary cases of YWBC as compared to unaffected young women may identify exosomes as potential targets for investigation into why YWBC, or subsets thereof like PPBC, are more prone to drug resistance, local recurrences and metastasis. The importance of the tumor microenvironment and immune system in breast cancer is increasingly identified as impacting prognosis and treatment benefit. Our data demonstrating altered stromal attributes of desmoplasia and immune suppressive milieu in preliminary human studies of PPBC supports an "exosome role" in mediating these events and if identified, also offer the potential for a biomarker to better identify the most "at risk" population among PPBC.

Aim 2: Determine if short-term drug intervention with anti-inflammatory agents in newly diagnosed young women with breast cancer alters the exosome presence, protein content or function, and correlate these exosome endpoints with parity status as in Aim 1. Whether these same exosome endpoints correlate with tumor Ki67, a relevant clinical marker, will also be evaluated.

2a. Using plasma samples obtained from our completed window of opportunity clinical trial (COMIRB 08-1040) within the YWBC Translational Program, we will determine if short term anti-inflammatory intervention altered circulating exosomes quantity, protein content or function using proteomic methods and cell culture models.

2b. Correlate the changes identified in the exosomes by patient status as being PPBC v non-PPBC and by whether there was a concomitant treatment induced reduction of breast cancer Ki67 expression, a validated marker of poorer prognosis in BC.

> **Innovation, Rationale, Impact:** Identification of an alteration in exosome quantity, protein content or function, either in promoting immune suppression and/or in tumor cell proliferation, migration, apoptosis

and invasive morphology in 3D culture, will identify potential mechanisms for exosome activity in YWBC. The ability to beneficially alter exosome function with clinical administration of COX-2 inhibiting drugs in humans is completely novel. Correlation of exosome changes with clinical outcomes and parity status may identify, for the first time that exosomes are present and may contribute to the poor prognosis of PPBC. Positive results would also support the ongoing investigation of exosomes as potential targets for therapy in PPBC patients whose prognosis remains poor despite current treatment advances, and/or identify a readily obtainable non-invasive marker of PPBC risk.

Eligibility: Cases from the three COMRIB protocols will be identified by review of our database (no PHI included) and selected based on their age, parity status, and the availability of collected sample to meet the experimental cohorts of interest for the two aims outlined above. There will be no subject contact or obtainment of new data or samples to complete the work as outlined in this protocol.

Patient Numbers: We have previously obtained blood and urine on 65 normal young female donors and 150 blood, tissue and urine samples from women ≤45 newly diagnosed with YWBC, untreated. From these samples, we have selected to start with twenty cases per cohort from which to isolate exosomes, balanced by PPBC to non-PPBC status, stage and clinical breast cancer subtype, as well as 20 normal age-matched controls. We anticipate that samples may need to be replaced with additional cases based on technical issues as the assays and experiments to be performed are new for our lab. We also have 22 cases of pre and post samples from women with newly diagnosed young onset breast cancer who were enrolled on protocol 08-1040 and exposed to celecoxib or no drug that will be utilized for Aim 2.

Research studies: All samples will be subjected to exosome isolation and content analyzed via standardized protocols. The isolated exosomes will under proteomic analysis and studied in cell culture assays, such as determination of proliferation, migration and invasion capacity of tumor cells after co-culture with exosomes. The exosomes will be analyzed for their effect on cellular morphology and apoptosis in 3D culture assays and studied in immune assays to determine their immune suppressive effects on human peripheral blood mononuclear cells.

Table of contents

1.0 Background and significance: YWBC and the Poor Prognosis of PABC: Breast cancer is the leading cancer diagnosis in young, premenopausal women and it has a higher incidence than the combined incidence of next five most frequent cancers in this age. SEER database 2012 statistics show 12% of all breast cancers are expected to occur under age 45, resulting in 27,000 young women's breast cases annually in the US alone. This rate places young women's breast cancer (YWBC) in the same general rate of incidence (cancers in females of all ages) as ovarian cancer (22,000) and pancreatic (21,000), and in excess of Hodgkin's lymphoma (4,100), cervical (12,000), and myeloid leukemia (10,500). An identifiable risk for breast cancer in younger women is a recent completed pregnancy. Pregnancy has a dual effect on breast cancer risk, conferring an immediate increased risk of developing breast cancer to all women regardless of age at pregnancy, and only later providing long term protection for women who are younger at first birth [1-6]. Further, older first-time mothers are at an elevated risk for breast cancer than younger first-time mothers (Fig 1). While there is no consensus on the definition of pregnancy-associated breast cancer (PABC), it is often limited in definition to cases diagnosed during pregnancy or very shortly afterward (<6 months postpartum), which represents a small fraction of all YWBC [7-9]. The rationale for focusing on diagnoses during pregnancy is logical, given the dominant role estrogen stimulation plays in breast cancer promotion and the fact that circulating levels of estrogen are increased ~40 fold during pregnancy [10-14]. However, large-scale, multi-institutional studies identify peak incidence of PABC at 5-7 years postpartum rather than during pregnancy (Fig 1) [3-5]. Furthermore, in our Colorado Cohort, relative risk for metastatic recurrence (HR 2.8) and death (HR 2.65) is significantly higher in young women diagnosed up to 5 years postpartum compared to nulliparous controls (Fig 2, VB, manuscript under revision). As shown in Fig 2, there is also a trend toward the increased risk of death persisting in the group diagnosed as late as 5-10 years postpartum. Our cohort is being expanded to better understand these later postpartum time points. Importantly, the effect size of the risk was not different when women were analyzed by each individual year post-partum up to year 5 (data not shown), demonstrating that risk for poor prognosis persists beyond the classical 6 month to 1 year postpartum cut-off. Moreover, the postpartum window has been identified as an independent predictor of poor prognosis, whereas pregnancy has not [15-19]. We argue that these data provide strong rationale for expansion of the definition of PABC to include postpartum cases. **Significance of problem:** To account for the poor outcomes experienced by women diagnosed at least as late as 5 years postpartum, the definition of PABC needs to be outcomes based and expanded to include postpartum cases. We anticipate a number of significant clinical implications as a consequence of including postpartum cases in the definition of PABC. The first is identification of a completed pregnancy within the last 5 years (or longer) as a new breast cancer risk factor for young women. Second, the number of women who may benefit from a prevention strategy targeted to involution is increased many fold over current estimates. For example, in a Norwegian cohort of 3034 YWBC cases, only 1% of the cases were diagnosed during pregnancy, while 44% were diagnosed within 6 years of a completed pregnancy (**Fig 3a**). Similar trends are observed in our University of Colorado cohort (**Fig 3b**). If PABC is re-defined by incidence and prognostic outcomes then up to 50% of ALL YWBC may be PABC. If half of young women's BC cases are in the setting of recent pregnancy and thus negatively influenced by the effect of involution on metastasis, the resultant 14,000 **high risk** PABC would still be greater in number than many of the other cancers listed. Therefore, targeting this highly vulnerable population of young mother's for prevention of PABC is both highly innovative and of substantial potential impact to the field.

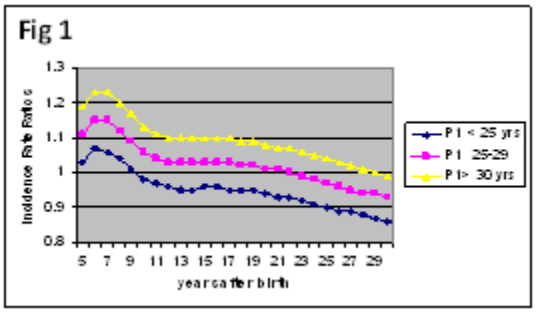


Figure 1: Evidence for a transient increase in breast cancer risk following pregnancy in uniparous women. Predicted incidence rate ratio of breast cancer for parous women by time since first birth, in subgroups by 'age at first birth'. For nulliparous women, the relative rate ratio is set to 1.0. In this cohort of 22,890 women with breast cancer, a transient increase in risk is seen up to 10 years post pregnancy for all age groups, but is highest in first time older mothers[38].

The Role of Postpartum Involution in Driving the Increased Risk for PABC:. Insight into why the postpartum window correlates strongly with poor prognosis has been obtained through rodent studies on postpartum mammary gland involution [20-22]. Work from our program and others demonstrate that postpartum involution utilizes wound healing and inflammatory programs to remodel the secretory-competent gland to a non-secretory state. Characteristics of provisional wound healing present in the involuting gland include accumulation of fibrillar collagen and the oncofetal extracellular matrix (ECM) protein tenascin-C, increases in matrix metalloproteinase (MMP) 2, 3, and 9, release of bioactive fragments of laminin and fibronectin with tumor activating properties, and an influx of alternatively activated macrophages with similarities to tumor-associated macrophages [21, 24-31] In tumor models, these same desmoplastic stromal attributes promote carcinogenesis and correlate with poor prognosis in breast cancer patients [21, 27, 31-37]. Based on this recent understanding of the cellular and molecular mechanisms of postpartum involution, we have proposed the 'involution-hypothesis' to account for the poor

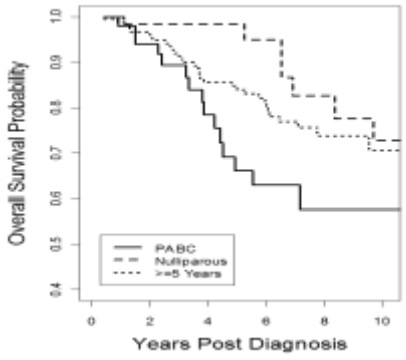


Figure 2: Evidence for decreased survival associated with PABC. Survival probability in PABC<5 (n=86), >=5 (n=172) and nulliparous (n=76) cases from the Colorado YWBC Cohort adjusted for tumor biologic subtype, clinical stage and year of diagnosis. PABC<5 have a markedly lower five-year survival of 65.8% in comparison with nulliparous cases, with a crude five-year survival of 98.0%. Cases >=5 had a crude five-year survival probability of 77.5%—intermediate to PABC<5 and nulliparous cases.

prognosis of PABC[7,28,38]. Specifically, we predict that wound healing attributes of the involuting gland drive promotion of pre-existing early stage lesions to overt, metastatic disease [27, 38]. To test this hypothesis, we developed rodent models for PABC where tumor cells are exposed to the mammary gland micro-environment in hosts with different reproductive states. Using a combination of fat pad and intraductal xenografts, and immune competent murine models, we consistently find that postpartum involution is tumor promotional[22]. Conversely, we find that pregnancy per se is not promotional in this model (unpublished data). These data reflect the human condition, where diagnosis in the postpartum window not pregnancy predicts outcomes for women with PABC[17, 18]. Recently, using our DCIS fat pad-xenograft model, a mechanism by which postpartum involution promotes tumor progression has been elucidated. In this model, fibrillar collagen, which is actively deposited during involution, induces cyclooxygenase-2 (COX-2) expression in tumor cells and drives COX-2 dependent tumor cell proliferation and invasion. At the same time as this tumorigenic microenvironment is developing, we identify the recruitment of "involution macrophages" with attributes of wound healing and tumor promotional M2 'alternatively' activated macrophages with expression of immunosuppressive cytokines, interleukin-1-(IL-10) ,macrophage chemoattractant protein-1(MCP-1) and IL-13 [21]. Additionally, we have identified, for the first time, that human involution also demonstrates an influx of CD45 leukocytes and specifically CD68 macrophages unique to the involution window, supporting the immune modulatory nature of human involution[21]. Based on these studies, we selected COX-2 as a target for intervention to modulate the tumor promotional and inflammatory involution microenvironment. Additional rationalization for targeting COX-2 comes from numerous epidemiologic studies [39-50]. In animal models, COX-2 overexpression induces mammary tumorigenesis [51] and in vitro inhibition of COX-2 reduces breast cancer cell proliferation, migration, and invasion [52]. Likewise, high COX-2 expression in breast tumor cells predicts infiltration of lung [47], bone [53], and brain [48]. In our preclinical postpartum BC model, we found that short-term NSAID treatment, with both non-specific ibuprophen and Cox-2 specific inhibitor celecoxib, limited to the 2 week window of mammary gland involution, sustainably reduces the ability of involution to promote tumor growth and metastasis [22]. We also found that both drugs reduced deposition of fibrillar collagen and ibuprophen reduced deposition of tenascin-C, to result in an ECM milieu with tumor-suppressive rather than activating properties (Fig4)[22, 54]. From this preclinical evidence, we moved forward into a translational Phase 0 Window of Opportunity human trial in newly diagnosed YWBC. The schema of this study is outlined in the research strategy and enrollment is completed. The primary aim of the parent study is to identify a decrease in Ki-67% index with the drug interventions, a known predictive marker for clinical benefit with short term intervention in breast cancer. Further, the biologic endpoints of identifying alterations in Cox-2 expression, M2- macrophage infiltration, collagen deposition, and other markers of desmoplasia and immune suppression at baseline and after drug intervention will provide supportive human data towards larger intervention clinical trials.

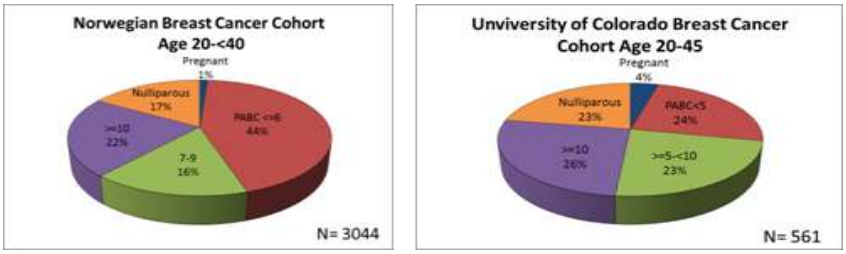


Figure 8: A recent pregnancy as a poor prognostic factor is common in young women with breast Cancer. a) Norwegian YWBC cohort separated by parity status shows 45% of women have a completed pregnancy within 6 years of diagnosis. b) University of Colorado YWBC cohort separated by parity status demonstrates similar trends.

<u>The role of exosomes in cancer as mediators of the tumor microenvironment and immune system:</u> Exosomes are microvesicles formed by internalization of the plasma membrane and subsequently released into the extracellular environment. The inward budding of the endosome membrane results in exosomes that contain cellular RNA, protein and DNA which are spontaneously released from various cells upon fusion with the plasma membrane. Exosomes are found ubiquitously in human body fluids, including plasma, malignant effusions and breast milk, and contain a wide variety of proteins, such as annexins, heat shock proteins, major histocompatability markers, co-stimulatory proteins, integrins, adhesion molecules, or metabolic enzymes [55]. Exosomes have been identified in the plasma of cancer patients at significantly increased levels as compared to unaffected persons, with the increased number of exosomes correlating with poorer prognosis and shorter survival[56, 57]. Clinically, exosomes have also been implicated in drug resistance [58, 59]. The role of exosomes in the tumor microenvironment and malignant behavior is expanding[75, 76]. They have been identified from multiple cancers as releasing TGFβ and thus being capable of inducing fibroblasts to express smooth muscle actin (αSMA) and differentiate into myofibroblasts with expression of SDF-1, VEGF, CCL5 and TGFβ. These multiple protumorigenic characteristics of

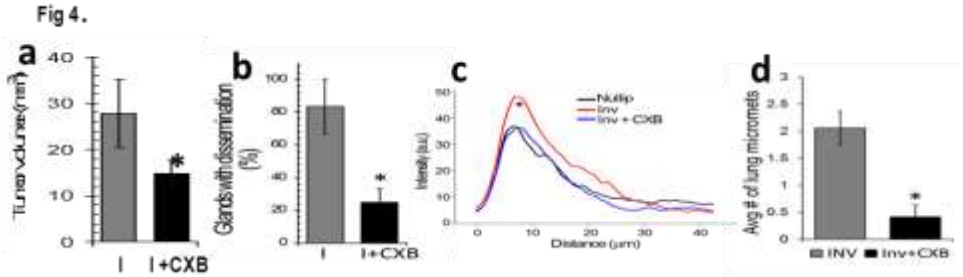


Fig 4. Celecoxib(CXB) abrogates the tumor promotional effects of involution and reverses mammary collagen to nulliparous levels. Mice were treated for 10 days with CXB during the window of postpartum involution. (a) Primary tumor volume reduced with CXB treatment at 3 weeks post-injection, *p=0.037, n=23 each cohort. (b) CXB reduces tumor cell dispersion that occurs during involution, p=0.0179, n=12 each cohort. (c) CXB reduces collagen deposition, as measured by SHG intensity versus distance from duct to levels observed in nulliparous animals (red) compared to drug treated nulliparous involuting mammary ducts (green) and nulliparous ducts (black), p<0.00001. (d) CXB reduced the average number of lung metastasis per animal compared with untreated involution, p=0.0002

exosomes support a role for exosomes in promoting stromal desmoplasia and driving cells toward a malignant phenotype[61, 60]. Likewise, tumor secreted exosomes home to local lymph nodes and other sites to prepare the metastatic niche for metastatic growth[62, 63]. In addition to the stromal effects of TGFβ releasing exosomes, they are also widely regarded as immunosuppressive[64].Exosomes excreted from various malignancies have immune suppressive effects, including inhibition of IL-2 induced T cell proliferation, inhibition of natural killer cell cytotoxicity[65], and induction of activated T lymphocyte apoptosis via FasL and TRAIL[66,67]. They also expand FoxP3+ regulatory T cells (Tregs) via TGFβ1 and IL-10 production[68], which in turn have increased FasL, IL-10, TGF-β1, granzyme B, perforin and increased suppressive capabilities. Furthermore, exosomes derived from malignant effusions can maintain Treg suppressive capacity and numbers[69]. Tumor secreted exosomes influence monocyte function and differentiation by inducing increased expression of IL10, TNFα, and IL-6 by monocytes[70] and driving their differentiation into putative myeloid derived suppressor cells (MDSC: $CD14^{+}HLA\text{-}DR^{-/low}$ cells in humans, CD11b+Gr1+ in mice). The resulting MDSC haveTGFβ dependent T cell suppression ability, increased production of IL-6 and VEGF, and promote tumor growth that in one model, was reversed by prostaglandin E2 (PGE-2) and TGFβ blockade[71]. Moreover, tumor derived exosomes inhibit myeloid cell differentiation to dendritic cells (DC) in a Myd88 dependent fashion, with the resultant skew toward increased MDSC leading to increased lung metastasis in the 4T1 murine mammary tumor model[72]. Tumor produced exosomes clearly modulate tumor immunosuppression, and macrophages can also produce exosomes that shuttle microRNA back into breast cancer cells and alter them to a more invasive phenotype[73]. Given that we have identified abundant macrophages in the involuting mammary gland the potential for exosomes playing a role in involution induced metastasis is great.

The combination of exosome isolation with advanced proteomic technologies to interrogate the proteome of the exosomes is starting to offer deeper insight to commonalities and uniqueness that can be identified across cell types in protein content and offer putative mechanisms behind their functional abilities. Our collaborator, M. Graner has identified exosomes with unique protein content from medulloblastoma. These exosomes contain high levels of hepatocyte nuclear factor 4alpha with potential tumor suppressor function, several proteins important in migration and proliferation and, interestingly, induce dichotomous dose-dependent T cell responses. In this model, low dose exosome exposure inhibits T cell gamma Interferon release while high dose exosome exposure increased the T cell response, suggesting a plasticity to exosomes[74]. Additional collaborators to our research, E. Eisenmesser and K.Hansen, in collaboration with the Graner lab, used a comprehensive approach of biochemical, biological, and spectroscopic methods to elucidate the stimulatory roles and potential mechanism of secreted exosomes from multiple tumor cells lines. Their results indicate that purified exosomes preferentially stimulate secretion of several pro-oncogenic factors in monocytic cells but only harbor limited activity with regard to epithelial cells. In addition, by using fluorescence microscopy, they have successfully visualized internalization of exosomes into the recipient cells within minutes. Finally, they identified, for the first time, a functional role for CD147/ extracellular matrix metalloproteinase inducer (EMMPRIN), which is a tumor cell surface protein that induces MMP and pro-inflammatory cytokine secretion, and has been found in tumor secreted exosomes. Functionally, the EMMPRIN containing exosomes were potent stimulators of MMP-9, IL-6, TGFβ1 and induced the secretion of extracellular EMMPRIN itself, all pro-oncogenic factors that drive immune evasion, tumor cell invasion, as well as, inflammation in the tumor microenvironment. (EE, manuscript in preparation)

The potential for exosome influence in the microenvironment of involution and PPBC: We have characterized, for the first time, the immune environment of the murine mammary gland across virgin, pregnant, lactating, involution and fully regressed parous states in an immunocompetent model and identified unique peaks of immune cell influx during involution for DC, monocytes, Tregs and more modestly CD4 Tcells(Fig5). We also isolated macrophages isolated from actively involuting glands that expressed significantly higher amounts of mannose receptor a M2 macrophage marker and allograft-inflammatory factor-1 (Aif-1) a tumor promotional cytokine, and showed they were capable of suppressing T cell activation *ex vivo*. Isolated involution myeloid cells also suppressed T cell activation and INF-γ production in *ex vivo* co-culture assays (unpublished data not shown) all supporting the involuting mammary gland as having an immune suppressive milieu in addition to the already identified desmoplastic microenvironment[77]. Interestingly the putative MDSC (CD11b+) subset in circulation from involution was suppressive as well, though not numerically increased from the other parity states (data not shown). These data pair with our recent characterization of young women's immune function from unaffected and newly diagnosed breast cancer cases. As expected, there is an increase in Tregs in the cancer cases, but unexpectedly, we identify similar numbers of myeloid derived suppressor cells in both populations, yet with increased T cell suppressive activity in the cancer cohort (Fig 6). To date, human MDSC data has linked increased numbers with increased suppression, [78-80] and we attribute the difference in our results to be accounted for by the robust size of our cohorts, the use of age-matched, gender-matched controls, and/or reflective of a true biologic difference. Our data on immune modulation during involution, taken with the emerging role for exosomes in cancer promotion and immune suppression, lead us to hypothesize that

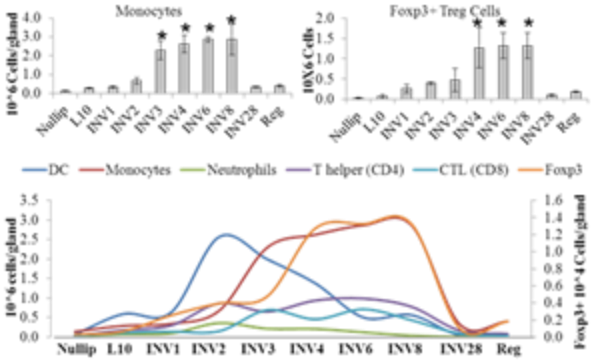


Fig 5. Involution demonstrates an increase in suppressive immune cells. Mammary gland digests from different time points of parity where subjected to FACs for phenotyping of infiltrating immune cells. A dominant pattern of monocyte and Tregs increase during mid involution without as dramatic an increase in other T cell subsets was identified, supportive of involution being an immunosuppressive milieu

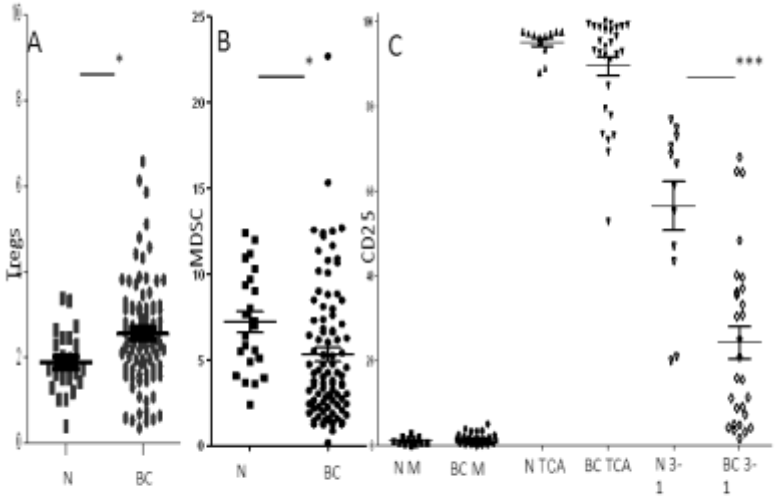


Figure 6 Characterization of an immune suppressive phenotype in YWBC. A. Significant increase in regulatory T cells as identified by FACS ($CD4^+CD25^{hi}FOXP3^+$) in YWBC (n=88) compared to normals (n=23). *p=.0131 **B.** MDSC ($Lin^{(-)}$ (CD3,CD14,CD19,CD20,CD56)HLA-$DR^-CD33^+CD11b^+$) are not different in normals (n=23) v. YWBC (n=101). *p=.0315 **C.** MDSCs from YWBC (n=27) are more suppressive (n=12). Suppression of T cell activation (TCA) by enriched CD11b+ MDSCs was assessed by FACS for CD25+ T cell levels. MDSCs were co-cultured at 3:1 with autologous T cells and CD3/CD28 stimulation beads for 96 hours. ***p=.0001

exosomes with pro-metastatic cargo are released from the actively involuting gland, enter the circulation, and influence tumor-microenvironment interactions, immune escape, and the metastatic niche. To begin to address this hypothesis, in this proposal, our objectives are to see whether exosomes with unique properties can be identified during involution, and are likewise present in women with PPBC. Specifically, we will characterize the protein content of involution exosomes, investigate their role in driving in vitro attributes of tumor cell invasiveness, and their ability to influence monocyte differentiation and promote Treg number and function. Importantly, we will determine whether exosomes can be blocked by PGE-2 inhibition in young women's breast cancer patients, supporting exosomes as a part of the tumor promotional attributes of involution that are modifiable by COX-2 inhibition. Complimentary murine studies will be performed as well. Our innovative hypothesis introduces the new paradigm that secreted exosomes unique to the life-window of postpartum breast involution contribute to the durable alteration of the involution microenvironment and its tumor promotional effects with potential to influence subsequent PPBC outcomes.

2.0 Hypothesis: We propose that postpartum breast involution results in the release of unique exosomes that enter circulation and have pro-tumorigenic interaction with tumor cells and the immune system. These unique exosomes will also be identifiable in post-partum breast cancer (PPBC) with altered protein composition and function and will correlate with known clinical markers of prognosis. Moreover, we predict that anti-inflammatory therapy targeting postpartum involution and/or the tumor microenvironment of PPBC will alter the circulating exosome composition and function, demonstrating a pro-tumorigenic, immunomodulatory role of exosomes in PPBC.

3.0 Specific Aims:

Aim 1. Characterize the circulating exosomes present in YWBC. Determine the type of circulating exosomes, protein content and function between unaffected young women and age-matched newly diagnosed cases of YWBC and correlate these findings with parity status (PPBC or non-PPBC) and known prognostic clinical tumor characteristics.

Innovation, Rationale and Impact: The identification of increased or unique circulating exosomes in primary cases of YWBC as compared to unaffected young women may identify exosomes as potential targets for investigation into why YWBC, or subsets thereof like PPBC, are more prone to drug resistance, local recurrences and metastasis. The importance of the tumor microenvironment and immune system in breast cancer is increasingly identified as impacting prognosis and treatment benefit. Our data demonstrating altered stromal attributes of desmoplasia and immune suppressive milieu in preliminary human studies of PPBC supports an "exosome role" in mediating these events and if identified, also offer the potential for a biomarker to better identify the most "at risk" population among PPBC.

Aim 2: Determine if short-term drug intervention with anti-inflammatory agents in newly diagnosed young women with breast cancer alters the exosome presence, protein content or function, and correlate these exosome endpoints with parity status as in Aim 2. Whether these same exosome endpoints correlate with tumor Ki67, a relevant clinical marker, will also be evaluated.

2a. Using plasma samples obtained from our completed window of opportunity clinical trial within the YWBC Translational Program, we will determine if short term anti-inflammatory intervention altered circulating exosomes quantity, protein content or function using proteomic methods and cell culture models.

2b. Correlate the changes identified in the exosomes by patient status as being PPBC v non-PPBC and by whether there was a concomitant treatment induced reduction of breast cancer Ki67 expression, a validated marker of poorer prognosis in BC.

Innovation, Rationale, Impact: Identification of an alteration in exosome quantity, protein content or function, either in promoting immune suppression and/or in tumor cell proliferation, migration, apoptosis and invasive morphology in 3D culture, will identify potential mechanisms for exosome activity in YWBC. The ability to beneficially alter exosome function with clinical administration of COX-2 inhibiting drugs in humans is completely novel. Correlation of exosome changes with clinical outcomes and parity status may identify, for the first time that exosomes are present and may contribute to the poor prognosis of PPBC. Positive results would also support the ongoing investigation of exosomes as potential targets for therapy in PPBC patients whose prognosis remains poor despite current treatment advances, and/or identify a readily obtainable non-invasive marker of PPBC risk

4.0 Eligibility

All cases previously enrolled to COMIRB protocols 08-1040, 09-0583 and 11-0357 will be considered as eligible for inclusion in this sample re-use protocol. Cases will be selected for inclusion in this work based on their age, parity status and available of needed plasma sample to meet the experimental cohorts as outlined. It is anticipated that only a subset of cases on 09-0583 and 11-0357 will be needed to meet the study endpoints and that all cases enrolled on 08-1040 who completed the study designated drug intervention or control prior to their surgery and on whom samples were obtained will be utilized. Full details of the inclusion and exclusion of the women enrolled in the parent protocols is available upon request to the PI.

5.0 Patient Numbers

We have previously obtained blood and urine on 65 normal young female donors and 150 blood, tissue and urine samples from women ≤45 newly diagnosed with YWBC, untreated. From these samples, we have selected to start with twenty cases from each experimental group which to isolate exosomes, balanced by PPBC to non-PPBC status, stage and clinical breast cancer subtype, as well as 20 normal age-matched controls. We anticipate that samples may need to be replaced with additional cases based on technical issues as the assays and experiments to be performed are new for our lab. We also have 22 cases of pre and post samples from women with newly diagnosed young onset breast cancer who were enrolled on protocol 08-1040 and exposed to celecoxib or no drug that will be utilized for Aim 2.

6.0 Immunologic Studies and research approach

Aim 1. Identify if YWBC, including the subsets of PPBC and non-PPBC, is characterized by an increase in circulating exosomes and if those exosomes have unique proteome content, and/or tumor promoting and immune suppressive function.

We will utilize the experimental cohorts of normal versus PABC versus non-PABC human plasma with samples kept individual to provide ability to correlate results with clinical prognostic data. Exosomes will be isolated and aliquoted to proteomic analysis and to cell culture assays to determine their impact on the human MCF10-DCIS.com cell line that mimics *ductal carcinoma in situ*. The MCF10.DCIS cell line is chosen as the best representative xenograft model of early stage disease, and we have previously identified that involution promotes these cells to acquire increased proliferation, invasion, and metastatic ability in vivo and in vitro[22]. An aliquot will also go for immune assays where dilutions of exosomes will be co-cultured with bone marrow derived donor cells and assayed for Treg, and monocytic/ putative MDSC induction. CD3+ selection for splenic T cells will be performed, then cells exposed to the various exosomes in standard Tcell culture assays to determine whether Tcell function is modulated by differing exosome levels. Assay details are outlined below.

Sample Acquisition: We have obtained blood and urine on 65 normal young female donors and 150 blood, tissue and urine samples from women ≤45 newly diagnosed with YWBC, untreated. Dr. Borges is the PI of both of these IRB approved protocols and all samples were processed identically. From these samples, we will select approximately twenty cases from which to isolate exosomes, balanced by PABC to non-PABC status, stage and clinical breast cancer subtype.

Anticipated results: PPBC will be associated with more circulating exosomes that demonstrate pro-metastatic attributes and function, as well as, immune modulatory abilities at differing levels. YWBC will have similar results in comparison to normal, but the overall differences will be more modest, similar to the immune profiling results identified for MDSCs.

Potential problems and alternatives: We have already successfully isolated exosomes from human plasma, so that is not a concern at present. We also have urine to use if quantitations are too low. If PPBC does not prove to have exosomes with unique attributes, we still feel these experiments will add to the relatively small published literature on primary human breast cancer exosomes. Detailed proteome analysis on primary samples will be revealing and functional analysis will identify potential roles for targeting or biomarker development of exosomes, even if not PPBC specific. We have chosen one cell line as the read out for our cell culture assays; however, the Borges lab has multiple cell lines with genomic profiling to the major breast cancer biologic subtypes that can be added as the project progresses.

Aim 2 will determine if a short-term drug intervention with anti-inflammatory agents in newly diagnosed YWB C alters the exosome number, protein content or function and will correlate these exosome endpoints with parity status as in Aim 1, as well as tumor Ki67 index, a known, predictive marker for clinical benefit. The objective of Aim 2 is to demonstrate that anti-inflammatory intervention in human post-partum breast cancer can reduce the tumor-promotional attributes of these cancers, in part, through modulation of the exosome profile. Our program recently completed a “Window of Opportunity” (WOO), Phase 0, randomized, open label, drug intervention study in YWBC patients with the anti-inflammatory agent, celecoxib versus control study [COMIRB 08-1040].. Subjects were recruited pre-surgery and completed 7 days or more of drug. Tissue, blood and urine were collected pre and post intervention. We will utilize the plasma samples collected on this trial, but not usedpreviously, to demonstrate that the circulating exosomes can be altered by Cox-2 inhibition, with a decrease of their tumor promotional attributes. **Tissue acquisition:** Patient consented to our IRB approved WOO study included future analysis of plasma samples for tumor promoting and immune modulating properties. Borges is the study PI. All samples are immediately available. **Anticipate outcomes:** 10 control and 10 celecoxib samples will be used with individualized results for comparison both pre and post intra-patient and also drug naïve versus drug exposed cohorts. PPBC cases will demonstrate greater sensitivity to reversal of exosome pro-metastatic and immune suppressive function with celecoxib than non PPBC, suggesting a Cox-2 dependent effect on exosomes occurs with PPBC as predicted from our animal models.

Problem and alternatives: The effect of Cox-2 inhibition may not be identifiable on circulating exosomes. In this case, we can expand enrollment for additional subjects and take fresh tissue (permitted in the protocol) for isolation of breast cancer exosomes in patients drug naïve versus exposed. We may then lose the pre and post intra-patient comparison due to size limitations of core samples, but if the exosomes are relatively consistent amongst cancer patients, then group comparison will be adequate. If breast cancer subtype is a confounding factor to exosome function or reversibility with Cox-2 inhibition in PPBC v non-PPBC, we may see confusing results, requiring expansion of the Aim 2 cohort to better delineate.

Exosome isolation and identification: Using plasma frozen at -80°C, thawed samples will be mixed 1:1 with PBS, filtered through a 0.45 µm filter and spun at 200,000 x g for 2 hours at 4°C using a Beckman L7-55 Ultracentrifuge and the Ti-70.1 rotor. Supernatants are removed and exosome enriched pellets re-suspended in radio-Immunoprecipitation assay buffer for western blot analysis or phosphate buffered saline for analysis with the Nanosight and functional assays. Size distributions and quantification of exosomes from each experimental group will be determined by measuring the rate of Brownian motion using a NanoSight LM10 system equipped with a fast video capture and particle-tracking software. Density gradient centrifugation for buoyant density determination, acetylcholinesterase activity determination, and transmission electron microscopy will be performed by our previously published methods[74].**Proteomic analysis**: Proteomic analysis of exosomes will be performed by collaborator Dr. Kirk Hansen, who has extensive experience in this area. **Cell culture assays:** All in vitro bioassays are generally run in quadruplicate and will be performed using exosomes from each experimental group Aim1-3. **Proliferation Assay**: Proliferation of the MCF10.DCIS cell line in response to co-culture with exosomes will be determined by standard KI-67% FACS staining of MCF10.DCIS cells after 48 hour exposure. **Migration Assay**: Human MCF10.DCIS cells will be set up at 100,000 cells per well in the upper well of a Boyden chamber (CytoSelect Cell Migration Assay, Cell BioLabs, Inc). Cells will be separated from the lower chamber, containing media that is either serum free, +10%FBS as positive control attractant, or with 50, 100 and 500ug/ml exosomes by an 8um pore size polycarbonate filter.

After 24-48 hours, the remaining non-motile cells will be removed from the upper chamber and the filter, with motile cells adhered, fixed with 10% NBF, stained with crystal violet, washed and number of migratory cells counted in 3 high power fields (40X), followed by an independent assessment using a dye extraction method. **Invasion Assay**: Invasion assays will be performed similarly with the exception that the filters are coated with high density reconstituted basement membrane (Matrigel) or collagen to assay for cellular invasion through ECM substratum. **3D culture assays:** Exosomes will be analyzed for their effect on cellular morphology, proliferation and apoptosis in 3D culture assays. Briefly, MCF10DCIS.com cells will be embedded in Matrigel + 10, 20, and 40% Collagen gels, previously shown to induce varying degrees of proliferation and invasive morphology. Cells will be incubated with exosomes for matrix embedding and analysis of 3D cultures for proliferation, apoptosis and morphology will be carried out as previously described[22]. **Immune assays:** Exosomes will be co-cultured with either BM derived progenitors or human donor PBMCs to determine induction rates for Treg or MDSCs, alterations of CD4:CD8 T cell ratios and alteration of macrophage Th1/Th2 polarization. Exosomes will be co-cultured with T cells and T cells + MDSCs in standard activation/suppression assays and then T cells analyzed by FACS for activation markers, CFSE-based proliferation assays, and γIFN release to determine suppression T cell function or enhancement of MDSC function. Supernatant will be collected for cytokine analysis using standard Luminex kits.

7.0 Concomitant medications

All enrolled subjects has recording of their concomitant medications at the time of sample collection, so that data is available for excluding any confounding drug effects from the research assays. No additional recording of data will occur

8.0 Study procedures

All data and tissue samples to be utilized in this research are on hand in the Young Women's Breast Cancer cohort, of which Borges is the PI. No PHI or identifiers will be used to conduct this research. No new data or subject contact will occur during this research. The data and samples to be used for this protocol do remain linked to the clinical information on the subjects through their medical record numbers and Borges holds this link. None of the lab personnel or collaborators have direct access to the clinical data, any PHI or to the link, nor will they ever be given access to them for any reason. The samples are kept linked for the purpose of eventually being able to correlate significant results in our longitudinal study of young women's breast cancer with patient outcomes of recurrence and survival in future research. In each of the three parent protocols from which the samples to be used in this protocol were obtained, enrolled subjects gave specific consent for their blood to be used for the study of exosomes and/or "circulating immune suppressive factors". Therefore, no new consenting of subjects is indicated for this work to proceed.

9.0 Adverse Event Reporting

Any adverse events related to the collection of the blood and urine samples would be reported under the specific protocol that the subject initially consented to. Any identified concern that the integrity of the security of our data system or risk that PHI may have been accessed by unauthorized persons will be immediately reported to COMIRB as per institutional guidelines.

10. Criteria for removal from study

Cases selected for inclusion in this protocol but deemed inadequate due to sample issues or failure of the research assays will be replaced by an additional matching case. Subjects enrolled to the parent protocols have the option of notifying us if the decide to withdraw consent. Any subject who withdraws consent and who has been slated for inclusion in this research protocol will be removed and replaced.

11.0 Statistical plan

Aim1: 20 samples from each of the normal, PPBC, and non-PPBC provide 80% power to detect a mean difference of 0.41 common within group standard deviation among the three groups using an F test with a 0.05 alpha level for each outcome described above. One-way ANOVA will be used to estimate and compare among (F test) and between groups (t-test).

Aim 2: 10 samples/experimental cohort provides 80% power to detect a mean of paired differences of 1.0 SD of differences with an alpha level of 0.05 using a two-sided paired t-test. 2x2 factorial design with 10 samples for each cohort provides 86% power to detect an effect size of 0.5 for drug or parity or interaction between the two using F test with a 0.05 alpha level . ANOVA will be used to estimate these effects and t-test for testing difference between effects. Spearman correlation coefficients and p-values will be calculated to correlate function and prognostic factors.

12. Data Quality Assurance and Monitoring of Study

All data to be used in this research protocol have been under ongoing review and audit by the University of Colorado Cancer Center Data Safety Monitoring Committee through the approved parent protocols from which the samples are derived. These three protocols have passed all audits with no outstanding concerns or queries. Regulatory approval for the three parent protocols remains in place and will continue to be maintained. Any concerns or actions against the parent protocols will also be reported to COMIRB, the DOD and HRPO in connection with any samples included in this research.

13. Ethical Aspects

The PI, Borges, attests that the three parent protocols under which the samples to be use in this research protocol were and continue to be conducted in full conformance with the principles of the "Declaration of Helsinki" and with laws and regulations of the United States of America. The studies have fully adhered to the principles outlined in the "Guidance for Good Clinical Practice" ICH Tripartite Guideline (January 1997) and the PI ensures that the basic principles of "Good Clinical Practice" as outlined in the current version of 21 CFR, subchapter D, part 312, "Responsibilities of Sponsors and Investigators", part 50, "Protection of Human Subjects", and part 56, "Institutional Review Boards" have been and will be adhered to.
The PI also attests that appropriate protocol was followed to obtain written informed consent from each subject participating in the three parent protocols, including adequate explanation of the aims, methods, anticipated benefits, and potential hazards of the studies. The investigator or designee also explained that the subjects were/are completely free to refuse to enter the study or to withdraw from it at any time for any reason. It is also the responsibility of the PI to assure that all Protected Health Information has been and will continue to be appropriately guarded to ensure subject confidentiality and that all potential subjects completed a "HIPPA B" form to allow the release of their identity to appropriately qualified protocol staff. Since the investigator on the study have a treatment relationship with the potential recruits, "HIPPA A" forms were required. All ICFs and Hippa forms are on file and will be provided to authorized parties upon request if needed.

14. Conditions for terminating the study

The study will be subject to termination if funding is withdrawn or at the discretion of the investigator.

15. Study documentation, CRFs and Record Keeping

There are no new patient data being collected. The clinical data to be utilized in this research already exists in the PIs password protected and secure RedCap database without PHI or identifiers available to the laboratory personnel. No PHI will be accessed as part of this research. The source documents and CRFs for the original data collections are maintained through the oversight of the individual protocols under which the samples to be used in this research protocol were obtained.

16. Publication of data

The results of this study may be published or presented at scientific meetings. The investigators will publish the data without the use of any information that would allow for individual subjects to be identified.

17. References

1. Lambe M, Hsieh C, Trichopoulos D, Ekbom A, Pavia M, Adami HO: Transient increase in the risk of breast cancer after giving birth, The New England journal of medicine 1994, 331:5-9
2. Robertson C, Primic-Zakelj M, Boyle P, Hsieh CC: Effect of parity and age at delivery on breast cancer risk in Slovenian women aged 25-54 years, International journal of cancer 1997, 73:1-9
3. Albrektsen G, Heuch I, Hansen S, Kvale G: Breast cancer risk by age at birth, time since birth and time intervals between births: exploring interaction effects, British journal of cancer 2005, 92:167-175
4. Albrektsen G, Heuch I, Kvale G: Further evidence of a dual effect of a completed pregnancy on breast cancer risk, Cancer Causes Control 1996, 7:487-488
5. Albrektsen G, Heuch I, Tretli S, Kvale G: Breast cancer incidence before age 55 in relation to parity and age at first and last births: a prospective study of one million Norwegian women, Epidemiology 1994, 5:604-611
6. Colditz GA, Frazier AL: Models of breast cancer show that risk is set by events of early life: prevention efforts must shift focus, Cancer Epidemiol Biomarkers Prev 1995, 4:567-571
7. Lyons TR, Schedin PJ, Borges VF: Pregnancy and breast cancer: when they collide, Journal of mammary gland biology and neoplasia 2009, 14:87-98
8. Vinatier E, Merlot B, Poncelet E, Collinet P, Vinatier D: [Breast cancer and pregnancy], Gynecol Obstet Fertil 2009, 37:495-503
9. Petrek JA: Pregnancy safety after breast cancer, Cancer 1994, 74:528-531
10. Vinatier E, Merlot B, Poncelet E, Collinet P, Vinatier D: Breast cancer during pregnancy, Eur J Obstet Gynecol Reprod Biol 2009, 147:9-14
11. Henderson BE, Pike MC, Casagrande JT: Breast cancer and the oestrogen window hypothesis, Lancet 1981, 2:363-364
12. Pike MC, Krailo MD, Henderson BE, Casagrande JT, Hoel DG: 'Hormonal' risk factors, 'breast tissue age' and the age-incidence of breast cancer, Nature 1983, 303:767-770
13. Pike MC, Spicer DV, Dahmoush L, Press MF: Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk, Epidemiol Rev 1993, 15:17-35
14. Tulchinsky D, Hobel CJ, Yeager E, Marshall JR: Plasma estrone, estradiol, estriol, progesterone, and 17-hydroxyprogesterone in human pregnancy. I. Normal pregnancy, American journal of obstetrics and gynecology 1972, 112:1095-1100
15. Bladstrom A, Anderson H, Olsson H: Worse survival in breast cancer among women with recent childbirth: results from a Swedish population-based register study, Clinical breast cancer 2003, 4:280-285
16. Whiteman MK, Hillis SD, Curtis KM, McDonald JA, Wingo PA, Marchbanks PA: Reproductive history and mortality after breast cancer diagnosis, Obstet Gynecol 2004, 104:146-154
17. Stensheim H, Moller B, van Dijk T, Fossa SD: Cause-specific survival for women diagnosed with cancer during pregnancy or lactation: a registry-based cohort study, J Clin Oncol 2009, 27:45-51
18. Johansson AL, Andersson TM, Hsieh CC, Cnattingius S, Lambe M: Increased Mortality in Women with Breast Cancer Detected during Pregnancy and Different Periods Postpartum, Cancer Epidemiol Biomarkers Prev 2011, 20:1865-1872
19. Beadle BM, Woodward WA, Middleton LP, Tereffe W, Strom EA, Litton JK, Meric-Bernstam F, Theriault RL, Buchholz TA, Perkins GH: The impact of pregnancy on breast cancer outcomes in women<or=35 years, Cancer 2009, 115:1174-1184
20. McDaniel SM, Rumer KK, Biroc SL, Metz RP, Singh M, Porter W, Schedin P: Remodeling of the mammary microenvironment after lactation promotes breast tumor cell metastasis, The American journal of pathology 2006, 168:608-620
21. O'Brien J, Lyons T, Monks J, Lucia MS, Wilson RS, Hines L, Man YG, Borges V, Schedin P: Alternatively activated macrophages and collagen remodeling characterize the postpartum involuting mammary gland across species, The American journal of pathology 2010, 176:1241-1255
22. Lyons TR, O'Brien J, Borges VF, Conklin MW, Keely PJ, Eliceiri KW, Marusyk A, Tan AC, Schedin P: Postpartum mammary gland involution drives progression of ductal carcinoma in situ through collagen and COX-2, Nat Med 2011, 17:1109-1115
24. Schedin P, Strange R, Mitrenga T, Wolfe P, Kaeck M: Fibronectin fragments induce MMP activity in mouse mammary epithelial cells: evidence for a role in mammary tissue remodeling, J Cell Sci 2000, 113 (Pt 5):795-806
25. Schedin P, Mitrenga T, McDaniel S, Kaeck M: Mammary ECM composition and function are altered by reproductive state, Molecular carcinogenesis 2004, 41:207-220
26. Schedin P, O'Brien J, Rudolph M, Stein T, Borges V: Microenvironment of the involuting mammary gland mediates mammary cancer progression, Journal of mammary gland biology and neoplasia 2007, 12:71-82
27. O'Brien J, Schedin P: Macrophages in breast cancer: do involution macrophages account for the poor prognosis of pregnancy-associated breast cancer?, Journal of mammary gland biology and neoplasia 2009, 14:145-157
28. Bemis LT, Schedin P: Reproductive state of rat mammary gland stroma modulates human breast cancer cell migration and invasion, Cancer research 2000, 60:3414-3418
29. Clarkson RW, Wayland MT, Lee J, Freeman T, Watson CJ: Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression, Breast Cancer Res 2004, 6:R92-109

30. Stein T, Morris JS, Davies CR, Weber-Hall SJ, Duffy MA, Heath VJ, Bell AK, Ferrier RK, Sandilands GP, Gusterson BA: Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3, Breast Cancer Res 2004, 6:R75-91
31. Stein T, Salomonis N, Nuyten DS, van de Vijver MJ, Gusterson BA: A mouse mammary gland involution mRNA signature identifies biological pathways potentially associated with breast cancer metastasis, Journal of mammary gland biology and neoplasia 2009, 14:99-116
32. Hancox RA, Allen MD, Holliday DL, Edwards DR, Pennington CJ, Guttery DS, Shaw JA, Walker RA, Pringle JH, Jones JL: Tumour-associated tenascin-C isoforms promote breast cancer cell invasion and growth by matrix metalloproteinase-dependent and independent mechanisms, Breast Cancer Res 2009, 11:R24
33. Ioachim E, Charchanti A, Briasoulis E, Karavasilis V, Tsanou H, Arvanitis DL, Agnantis NJ, Pavlidis N: Immunohistochemical expression of extracellular matrix components tenascin, fibronectin, collagen type IV and laminin in breast cancer: their prognostic value and role in tumour invasion and progression, Eur J Cancer 2002, 38:2362-2370
34. Levy P, Ripoche H, Laurendeau I, Lazar V, Ortonne N, Parfait B, Leroy K, Wechsler J, Salmon I, Wolkenstein P, Dessen P, Vidaud M, Vidaud D, Bieche I: Microarray-based identification of tenascin C and tenascin XB, genes possibly involved in tumorigenesis associated with neurofibromatosis type 1, Clin Cancer Res 2007, 13:398-407
35. Taraseviciute A, Vincent BT, Schedin P, Jones PL: Quantitative analysis of three-dimensional human mammary epithelial tissue architecture reveals a role for tenascin-C in regulating c-met function, The American journal of pathology 2010, 176:827-838
36. Conklin MW, Eickhoff JC, Riching KM, Pehlke CA, Eliceiri KW, Provenzano PP, Friedl A, Keely PJ: Aligned collagen is a prognostic signature for survival in human breast carcinoma, The American journal of pathology 2011, 178:1221-1232
37. Schedin P, Keely PJ: Mammary gland ECM remodeling, stiffness, and mechanosignaling in normal development and tumor progression, Cold Spring Harb Perspect Biol 2011, 3:a003228
38. Schedin P: Pregnancy-associated breast cancer and metastasis, Nature reviews 2006, 6:281-291
39. Singh-Ranger G, Salhab M, Mokbel K: The role of cyclooxygenase-2 in breast cancer: review, Breast Cancer Res Treat 2008, 109:189-198
40. Mazhar D, Ang R, Waxman J: COX inhibitors and breast cancer, British journal of cancer 2006, 94:346-350
41. Kwan ML, Habel LA, Slattery ML, Caan B: NSAIDs and breast cancer recurrence in a prospective cohort study, Cancer Causes Control 2007, 18:613-620
42. Holmes MD, Chen WY, Li L, Hertzmark E, Spiegelman D, Hankinson SE: Aspirin intake and survival after breast cancer, J Clin Oncol 2010, 28:1467-1472
43. Ristimaki A, Sivula A, Lundin J, Lundin M, Salminen T, Haglund C, Joensuu H, Isola J: Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer, Cancer research 2002, 62:632-635
44. Denkert C, Winzer KJ, Muller BM, Weichert W, Pest S, Kobel M, Kristiansen G, Reles A, Siegert A, Guski H, Hauptmann S: Elevated expression of cyclooxygenase-2 is a negative prognostic factor for disease free survival and overall survival in patients with breast carcinoma, Cancer 2003, 97:2978-2987
45. Spizzo G, Gastl G, Wolf D, Gunsilius E, Steurer M, Fong D, Amberger A, Margreiter R, Obrist P: Correlation of COX-2 and Ep-CAM overexpression in human invasive breast cancer and its impact on survival, British journal of cancer 2003, 88:574-578
46. Howe LR: Inflammation and breast cancer. Cyclooxygenase/prostaglandin signaling and breast cancer, Breast Cancer Res 2007, 9:210
47. Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, Viale A, Olshen AB, Gerald WL, Massague J: Genes that mediate breast cancer metastasis to lung, Nature 2005, 436:518-524
48. Bos PD, Zhang XH, Nadal C, Shu W, Gomis RR, Nguyen DX, Minn AJ, van de Vijver MJ, Gerald WL, Foekens JA, Massague J: Genes that mediate breast cancer metastasis to the brain, Nature 2009, 459:1005-1009
49. Gauthier ML, Berman HK, Miller C, Kozakeiwicz K, Chew K, Moore D, Rabban J, Chen YY, Kerlikowske K, Tlsty TD: Abrogated response to cellular stress identifies DCIS associated with subsequent tumor events and defines basal-like breast tumors, Cancer cell 2007, 12:479-491
50. Visscher DW, Pankratz VS, Santisteban M, Reynolds C, Ristimaki A, Vierkant RA, Lingle WL, Frost MH, Hartmann LC: Association between cyclooxygenase-2 expression in atypical hyperplasia and risk of breast cancer, Journal of the National Cancer Institute 2008, 100:421-427
51. Liu CH, Chang SH, Narko K, Trifan OC, Wu MT, Smith E, Haudenschild C, Lane TF, Hla T: Overexpression of cyclooxygenase-2 is sufficient to induce tumorigenesis in transgenic mice, The Journal of biological chemistry 2001, 276:18563-18569
52. Larkins TL, Nowell M, Singh S, Sanford GL: Inhibition of cyclooxygenase-2 decreases breast cancer cell motility, invasion and matrix metalloproteinase expression, BMC cancer 2006, 6:181
53. Singh B, Berry JA, Shoher A, Ayers GD, Wei C, Lucci A: COX-2 involvement in breast cancer metastasis to bone, Oncogene 2007, 26:3789-3796
54. O'Brien J, Hansen K, Barkan D, Green J, Schedin P: Non-steroidal anti-inflammatory drugs target the pro-tumorigenic extracellular matrix of the postpartum mammary gland, Int J Dev Biol 2011, 55:745-755
55. Ge R, Tan E, Sharghi-Namini S, Asada HH: Exosomes in Cancer Microenvironment and Beyond: have we Overlooked these Extracellular Messengers?, Cancer Microenviron 2012,

56. Silva J, Garcia V, Rodriguez M, Compte M, Cisneros E, Veguillas P, Garcia JM, Dominguez G, Campos-Martin Y, Cuevas J, Pena C, Herrera M, Diaz R, Mohammed N, Bonilla F: Analysis of exosome release and its prognostic value in human colorectal cancer, Genes Chromosomes Cancer 2012, 51:409-418
57. Bergmann C, Strauss L, Wieckowski E, Czystowska M, Albers A, Wang Y, Zeidler R, Lang S, Whiteside TL: Tumor-derived microvesicles in sera of patients with head and neck cancer and their role in tumor progression, Head Neck 2009, 31:371-380
58 Safaei R, Larson BJ, Cheng TC, Gibson MA, Otani S, Naerdemann W, Howell SB: Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells, Mol Cancer Ther 2005, 4:1595-1604
59. Hercpetin
60. Webber J, Steadman R, Mason MD, Tabi Z, Clayton A: Cancer exosomes trigger fibroblast to myofibroblast differentiation, Cancer Res 2010, 70:9621-9630
61. Cho JA, Park H, Lim EH, Lee KW: Exosomes from breast cancer cells can convert adipose tissue-derived mesenchymal stem cells into myofibroblast-like cells, Int J Oncol 2012, 40:130-138
62 Hood JL, San RS, Wickline SA: Exosomes released by melanoma cells prepare sentinel lymph nodes for tumor metastasis, Cancer Res 2011, 71:3792-3801
63. Jung T, Castellana D, Klingbeil P, Cuesta Hernandez I, Vitacolonna M, Orlicky DJ, Roffler SR, Brodt P, Zoller M: CD44v6 dependence of premetastatic niche preparation by exosomes, Neoplasia 2009, 11:1093-1105
64. Huber V, Filipazzi P, Iero M, Fais S, Rivoltini I. (2008) More insights into the immunosuppresive potential of tumor exosomes, J Transl Med 6:63
65. Clayton A, Mitchell JP, Court J, Mason MD, Tabi Z: Human tumor-derived exosomes selectively impair lymphocyte responses to interleukin-2, Cancer Res 2007, 67:7458-7466
66 Andreola G, Rivoltini L, Castelli C, Huber V, Perego P, Deho P, Squarcina P, Accornero P, Lozupone F, Lugini L, Stringaro A, Molinari A, Arancia G, Gentile M, Parmiani G, Fais S: Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles, J Exp Med 2002, 195:1303-1316
67. Huber V, Fais S, Iero M, Lugini L, Canese P, Squarcina P, Zaccheddu A, Colone M, Arancia G, Gentile M, Seregni E, Valenti R, Ballabio G, Belli F, Leo E, Parmiani G, Rivoltini L: Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: role in immune escape, Gastroenterology 2005, 128:1796-1804
68. Szajnik M, Czystowska M, Szczepanski MJ, Mandapathil M, Whiteside TL: Tumor-derived microvesicles induce, expand and up-regulate biological activities of human regulatory T cells (Treg), PLoS One 2010, 5:e11469
69 Wada J, Onishi H, Suzuki H, Yamasaki A, Nagai S, Morisaki T, Katano M: Surface-bound TGF-beta1 on effusion-derived exosomes participates in maintenance of number and suppressive function of regulatory T-cells in malignant effusions, Anticancer Res 2010, 30:3747-3757
70 Valenti R, Huber V, Filipazzi P, Pilla L, Sovena G, Villa A, Corbelli A, Fais S, Parmiani G, Rivoltini L: Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-beta-mediated suppressive activity on T lymphocytes, Cancer Res 2006, 66:9290-9298
71 Xiang X, Poliakov A, Liu C, Liu Y, Deng ZB, Wang J, Cheng Z, Shah SV, Wang GJ, Zhang L, Grizzle WE, Mobley J, Zhang HG: Induction of myeloid-derived suppressor cells by tumor exosomes, Int J Cancer 2009, 124:2621-2633
72 Liu Y, Xiang X, Zhuang X, Zhang S, Liu C, Cheng Z, Michalek S, Grizzle W, Zhang HG: Contribution of MyD88 to the tumor exosome-mediated induction of myeloid derived suppressor cells, Am J Pathol 2010, 176:2490-2499
73 Yang M, Chen J, Su F, Yu B, Lin L, Liu Y, Huang JD, Song E: Microvesicles secreted by macrophages shuttle invasion-potentiating microRNAs into breast cancer cells, Mol Cancer 2011, 10:117
74 Eppel
75 Filipazzi P, Burdek M, Villa A, Rivoltini L, Huber V: Recent advances on the role of tumor exosomes in immunosuppression and disease progression, Semin Cancer Biol 2012, 22:342-349
76. Iero M, Valenti R, Huber V, Filipazzi P, Parmiani G, Fais S, Rivoltini L: Tumour-released exosomes and their implications in cancer immunity, Cell Death Differ 2008, 15:80-88